STUDIES ON HYDROCARBON-UTILIZING MICRO-ORGANISMS

by

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CONTENTS

SUMMARY

Two aspects of the microbial utilization of n-alkanes were studied: the utilization of a liquid n-alkane fraction by yeasts, and the utilization of C_2 - C_4 gaseous n-alkanes by bacteria.

Numerous yeast strains were isolated from the environment and classified as Torulopsis and Rhodotorula species. The basal salts medium used in enrichments, was optimised by adjustment of H^+ ion and trace elements concentrations, and by supplementation with yeast extract, which provided the essential growth factor, folic acid; and also assisted initial hydrocarbon utilization by rendering the substrate more available to the cells.

Physical contact between cell and substrate was found to be essential for growth. In batch culture, growth rate was linear and dependent upon the surface area of substrate available. Attempts at growing the yeasts in continuous culture were unsuccessful, since shearing forces in the chemostat prevented attachment to the substrate. The industrial implications of these findings were discussed with a view to biomass production and fermentor design.

The protein content of hydrocarbon-grown Torulopsis cells was found to be 8% (w/w) higher than similarly grown Candida. The Rhodotorula isolates had a low protein content.

Isolation of gaseous hydrocarbon-utilizing bacteria

yielded a variety of organisms, whose morphological, and some physiological and biochemical properties were studied. Hydrogen autotrophy and methane utilization were found to be absent. After selection of two suitable Nocardia strains, growth rates on the gases were studied at different partial pressures and temperature. Growth rates and substrate preference were found to increase with carbon number. In closed batch culture, early growth was exponential, but later became linear as substrate became limiting.

Apparatus for growing bacteria on gaseous substrates in continuous culture was designed, but the Nocardia isolates were ruptured by the shearing forces within the chemostat.

Growth yields were greater (47% w/w) with gases than with the corresponding primary alcohols or methyl ketones. Adaption studies suggested that the site of oxygen insertion in the hydrocarbon was at the C_1-position.

Flame-ionisation techniques were developed to measure transfer rates of gases from water to air; and also to measure the concentration of methane in aqueous solution. Gas/liquid chromatography with flame-ionisation detection was used to estimate concentrations of C_2 - C_4 alcohols, aldehydes, methyl ketones and acids in culture media.

It was concluded that bacterial biomass production from C_2 - C_4 n-alkanes was economically impracticable.

INTRODUCTION

Biochemistry of Hydrocarbon Metabolism

Initial Attack

It is generally agreed that the initial step in the enzymic attack upon the hydrocarbon molecule is the insertion of atmospheric oxygen. With n-alkanes, one of the terminal methyl groups is oxidised. Stewart, Kallio, Stevenson, Jones and Schissler (1959) isolated cetyl palmitate from a Gram-negative coccus growing on n-hexadecane in an atmosphere enriched with O_2^{18}, and found large amounts of O^{18} were incorporated into this wax. Leadbetter and Foster (1959) detected O^{18} in the cellular material of a bacterium grown on ethane and propane in an atmosphere containing O_2^{18}. Baptist, Gholson and Coon (1963) detected n-octanol and octaldehyde as products of n-octane from an enzyme system extracted from a n-hexane-grown pseudomonad.

The actual mechanism of oxygen incorporation into the methyl group has not been elucidated. The following possible mechanisms have been suggested.

1-alkyl Hydroperoxide Formation

The suggested reaction is:

$$R.CH_2CH_3 + O_2 \longrightarrow R.CH_2.CH_2 - OOH \text{ (1-alkyl hydroperoxide)}$$

$$R.CH_2.CH_2 - OOH + NADH \xrightarrow{H+} R.CH_2.CH_2 - OH + NAD + H_2O$$

Such a mechanism would explain the results of O^{18} incorporation experiments of Stewart *et al.* (1959). Finnerty, Kallio, Klimstra and Wawzonek (1962) reported that the reduction of the 1-alkyl hydroperoxides of n-C_{12}, C_{14}, C_{16} and C_{18} alkanes occurred at rates which suggested that they might be intermediates. These workers also reported ester production from 1-alkyl hydroperoxides. McKenna and Kallio (1965) suggested that the reduction of cumene hydroperoxide by bacteria (Updegroff and Bovey, 1958) proceeded at a rate sufficient to make hydroperoxides possible intermediates in n-alkane oxidation. McKenna and Kallio (1965) (reporting their unpublished observations) found that n-hexadecane-grown *Micrococcus cerifi-cans* degraded 1-hexadecyl hydroperoxide to produce substances inseparable from n-hexadecanol and palmitic acid by thin layer chromatography.

Mixed Function Oxidase Mechanism

$$RCH_2.CH_3 + O_2 + NADH + H^+ \longrightarrow RCH_2.CH_2OH + NAD + H_2O$$

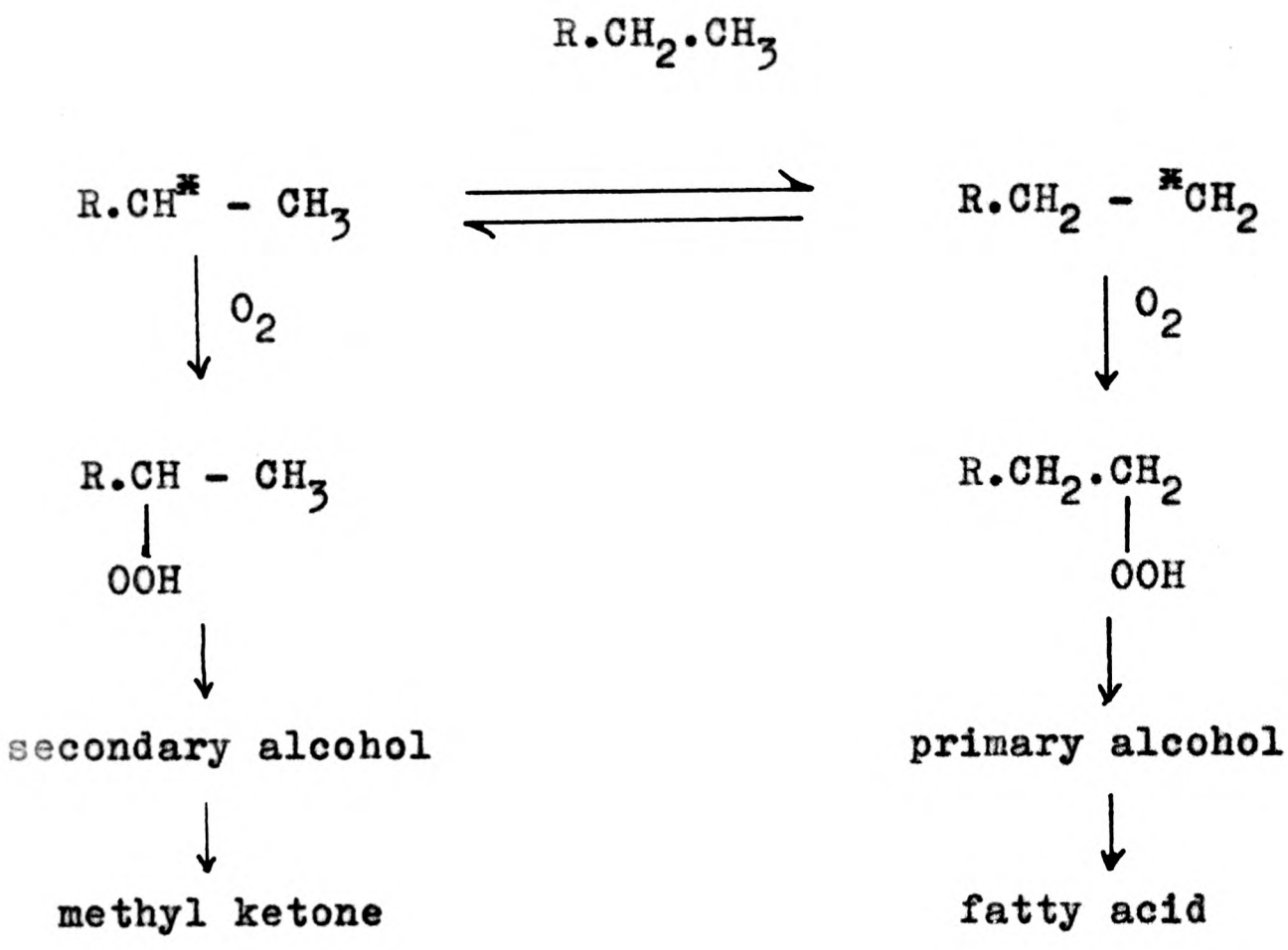
Baptist *et al.* (1963) and Gholson, Baptist and Coon (1963) isolated two enzyme systems from a n-C_6-grown pseudomonad, which, when combined, produced n-octanol and octaldehyde from n-octane. The initial attack on the alkane required NADH or NAD, and one of the two systems required ascorbate for stabilisation, while the other had a requirement for Fe^{++} or Fe^{+++} ions. The amount of aldehyde produced depended upon the amounts of aldehyde dehydrogenase and NADH oxidase present.

his results. The results of Thijsse and van der Linden (1963) would also tend to suggest that this was not a general mechanism. They found that heptane-grown cells oxidised 1-heptene in the C_7 position forming 6-heptenaic acid which would imply that hept-1-ene was not an intermediate in heptane oxidation.

Leadbetter and Foster (1960), in a co-oxidation experiment with Ps. methanica growing on methane and supplied with hexadeuteroethane, showed that the acetic acid produced could not have been formed via ethylene. In a similar type of experiment, by Lukins and Foster (1963), acetone production from propane by Mycobacterium smegmatis growing in D_2O enriched water, was shown not to proceed via propylene. Thus it would appear that the metabolism of gaseous hydrocarbons does not occur by a sequence involving olefin formation. McKenna and Kallio (1965) suggested that from thermodynamic considerations, the reaction postulated by Senez would not allow quantitative reduction of NAD.

Recent findings of Abbot and Casida (1965) have shown that the observation of Senez may not be as rare as was first supposed. These workers detected the production of 7-hexadecene from n-hexadecane by glucose-grown cells of Nocardia salmonicolor. They claim that this was not a reaction of the enzymes of fatty acid dehydrogenation, since no unsaturated fatty acids were detected when glucose-grown cells were incubated with stearic or palmitic acids. As well as

Figure 1 **Free Radical Mechanism**



7-hexadecene being formed, 8- and 6-hexadecene were found in lesser amounts.

Wagner, Zahn and Buhring (1967) detected 1-hexadecene from a variety of organisms grown on n-hexadecane. The organisms used were Mycobacterium phlei, Micrococcus cerificans, Nocardia, Pseudomonas and Rhodotorula sp. A cell-free extract of the Nocardia sp. also formed this olefin.

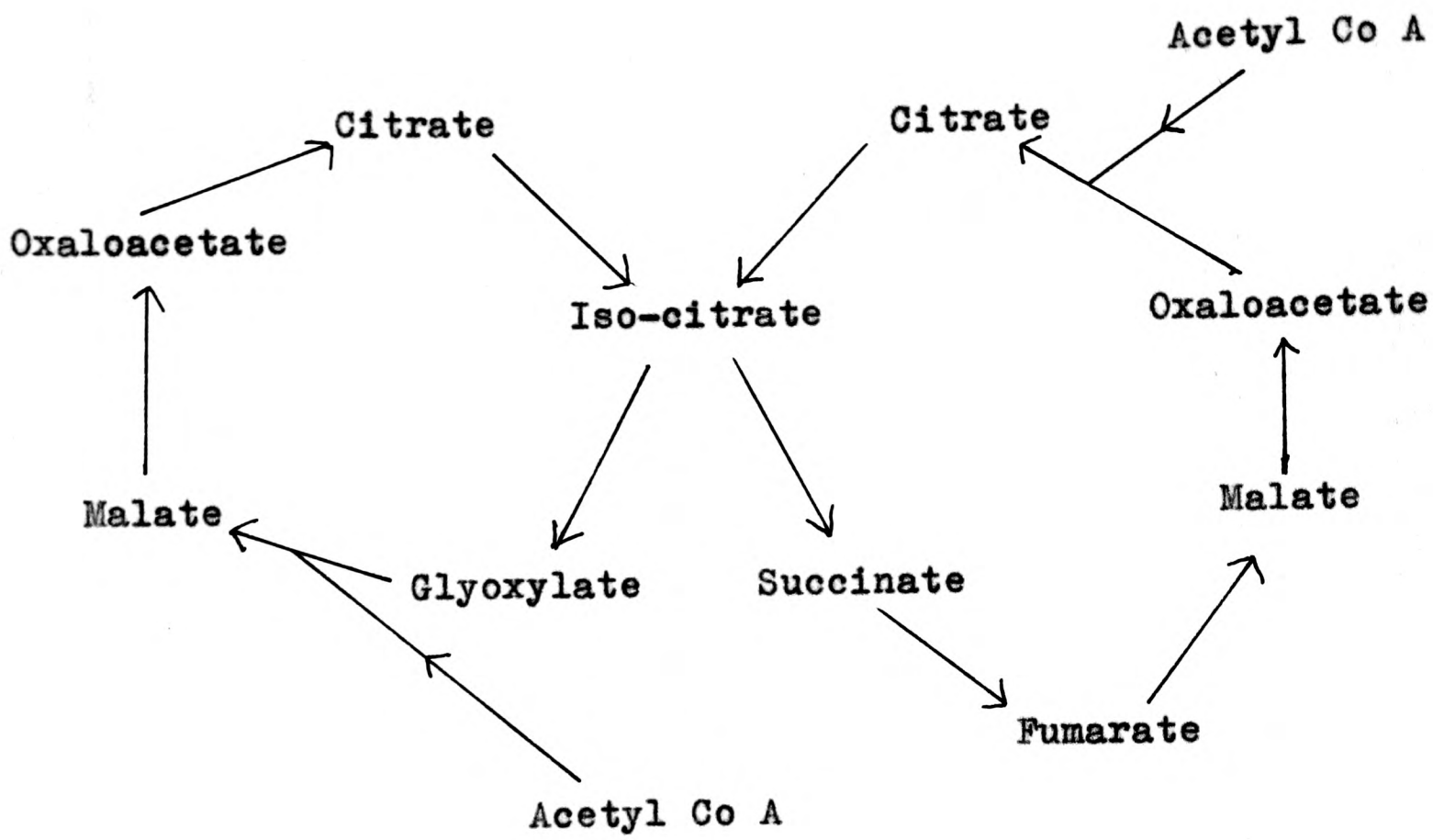
Recent improvements in identification techniques show that a desaturation of the alkane is more common than was once supposed, but the significance of this pathway in hydrocarbon metabolism remains obscure.

Free Radical Mechanism

This was suggested by Leadbetter and Foster (1960) to account for the appearance of methyl ketones as the products of co-oxidation of propane and n-butane by Pseudomonas methanica (Fig. 1). They suggested that oxygen insertion may be by 1- or 2-alkyl hydroperoxide formation, but no evidence for this was given.

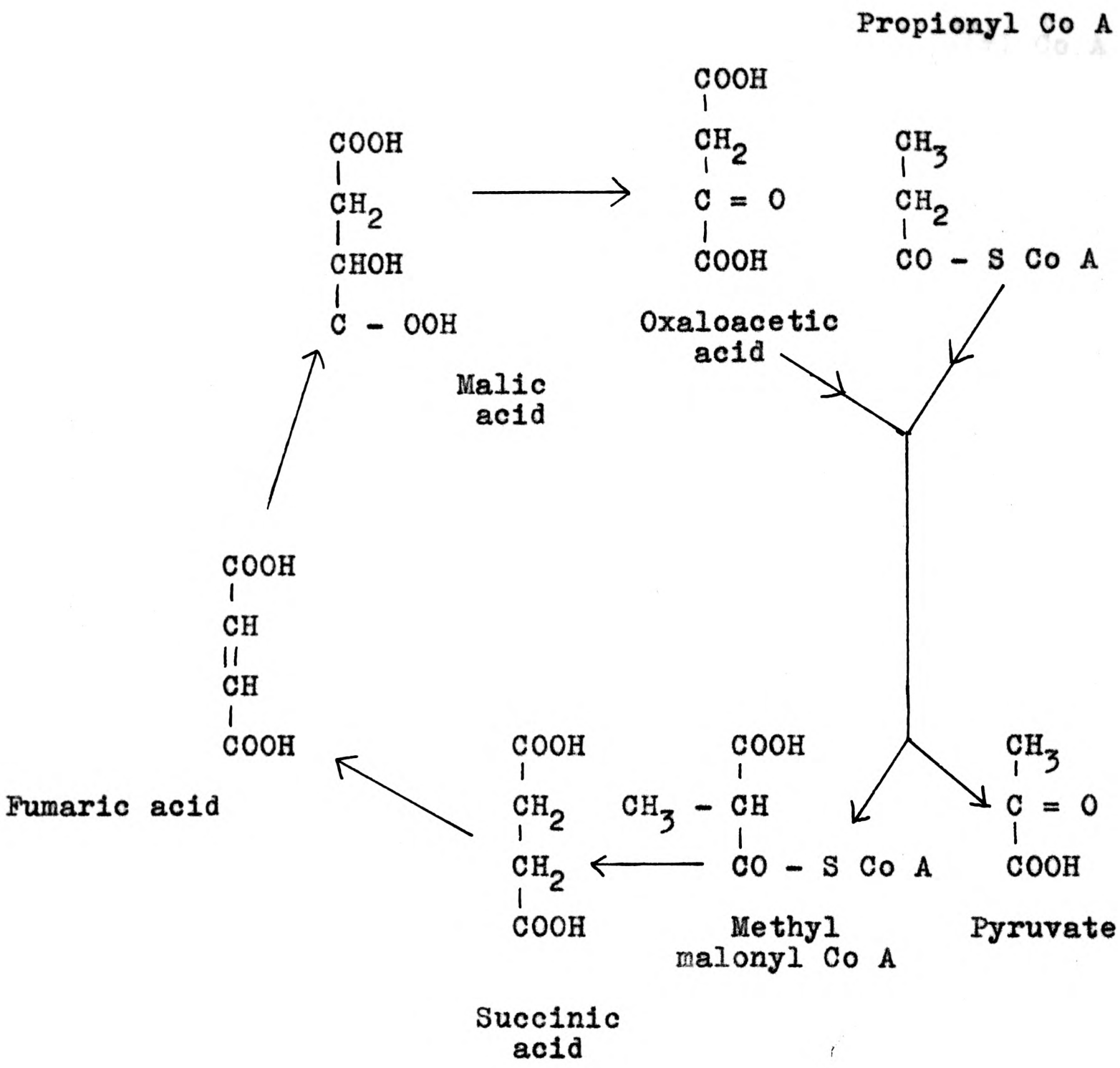
From this review of the possible modes of oxygen insertion into the n-alkane molecule, it is apparent that no conclusive evidence has been presented. It may be that no generalisation can be made, and that the mechanism may depend upon the chain length of the alkane and the micro-organism itself.

Figure 2 **Acetate Metabolism**



Glyoxylate By-pass Citric Acid Cycle

Figure 3 **Propionate Metabolism**



Subsequent Degradation of n-Alkanes

It is generally thought that the first product of n-alkane oxidation is a primary alcohol of the same carbon number as the substrate (Baptist et al., 1963). Cells adapted to growth on n-alkanes were shown to be simultaneously adapted to the corresponding alcohol, aldehyde or fatty acid. The fatty acid formed from the oxidation of the alcohol is further oxidised by the process of β -oxidation (Thijsse and van der Linden, 1958). (The metabolism of these two carbon fragments is shown in Fig. 2). The Mycobacterium rhodochrous of Fredricks (1967) was shown to produce fatty acids of even carbon numbers from C_2 to C_{10} during the β -oxidation of n-decane. In a similar type of experiment Heringa, Huybregtse and van der Linden (1961) showed that the degradation of n-C_6 and C_7 alkanes by Pseudomonas aeruginosa was by β -oxidation and that decarboxylation of fatty acids did not occur. Presumably the propionic acid formed by β -oxidation of an odd carbon numbered alkane is metabolised by a sequence as shown in Fig. 3. The enzymes for such a mechanism would not appear to be present during the oxidation of even numbered alkanes, since Thijsse and van der Linden (1963) found propionic acid accumulation during n-heptane oxidation by cells adapted to n-hexane. The synthesis of induced enzymes was inhibited by chloromphenicol.

There are reports of enzymic attack at both ends of the alkane molecule. Kester and Foster (1963) described the formation of the corresponding monoic-, ω -hydroxy monoic- and

dioic-acids from a series of n-alkanes from C_{10} to C_{14}. These observations were made using a corynebacter strain. Ali Khan, Hall and Robinson (1963, 1964), identified n-octanedioic acid in a culture of a n-hexane-grown pseudomonad supplied with n-octane. Ogino, Yano, Tamura and Arima (1965) detected dicarboxylic acids in cultures of a Pitchia species growing on n-C_{11} and n-C_{12} alkanes. It may be that diterminal oxidation occurs more frequently than is generally realised, since the intermediates may fail to accumulate in detectable quantities. Whether β -oxidation proceeds from both ends of the molecule is unknown, but such a sequence would favour more rapid utilization of substrate.

Methyl Ketone Production and Metabolism

Two mechanisms exist for the biological production of methyl ketones. In one the ketone formed has one carbon atom less than the original substrate as a result of decarboxylation of a β -keto acid (Franke and Heinen, 1958; Thijsse, 1964). The second mechanism, with which there is no reduction in the substrate carbon number, is considered in this report.

Leadbetter and Foster (1960), in co-oxidation studies, detected methyl ketones produced from short chain n-alkanes. Lukins (1962), using mycobacteria growing on short chain alkanes, found methyl ketones of n-C_3 to n-C_6 alkanes. He suspected that the methyl ketone of n-undecane was also produced, and suggested that methyl ketones may be produced

Figure 4 Acetone Metabolism

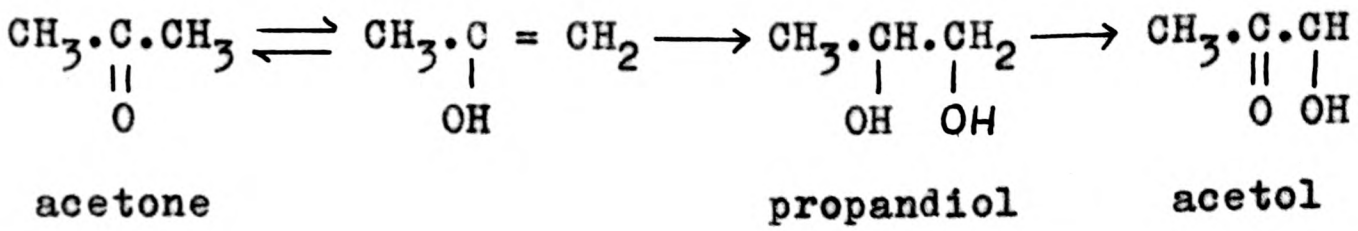


Figure 5 Acetone Metabolism

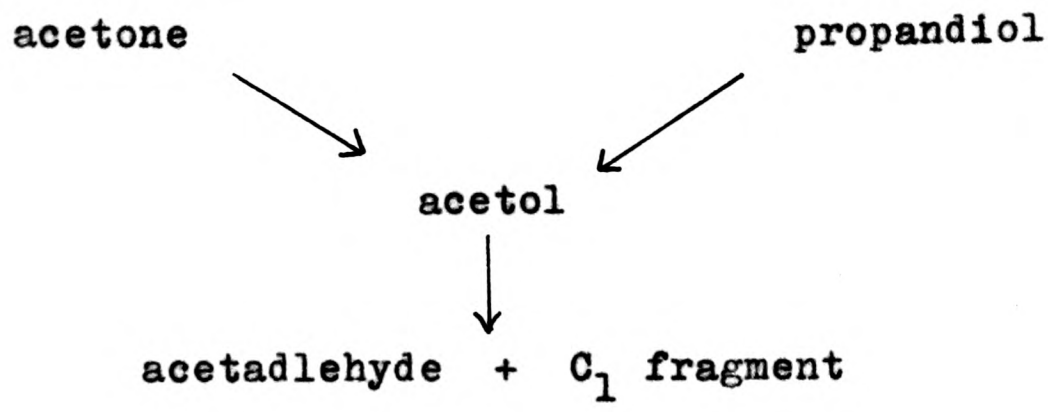


Figure 6 Methyl Ketone Metabolism

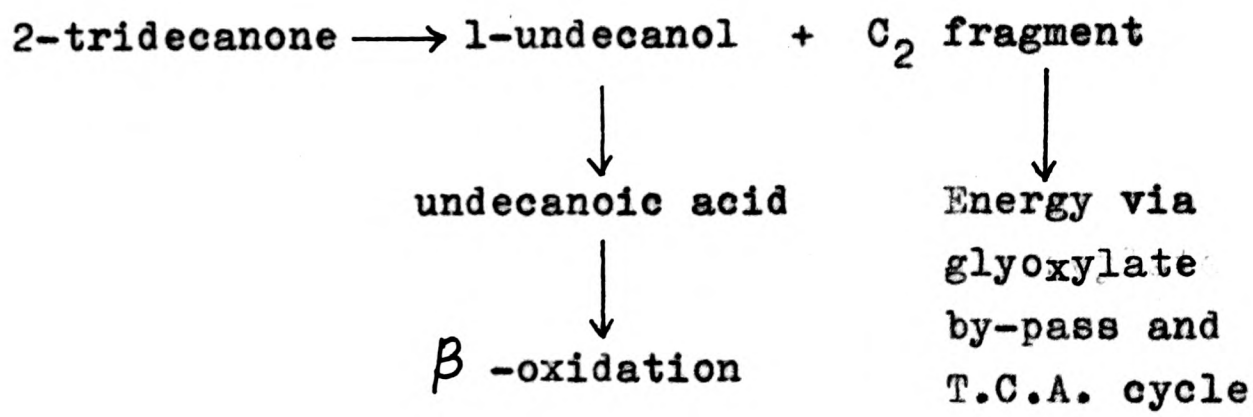
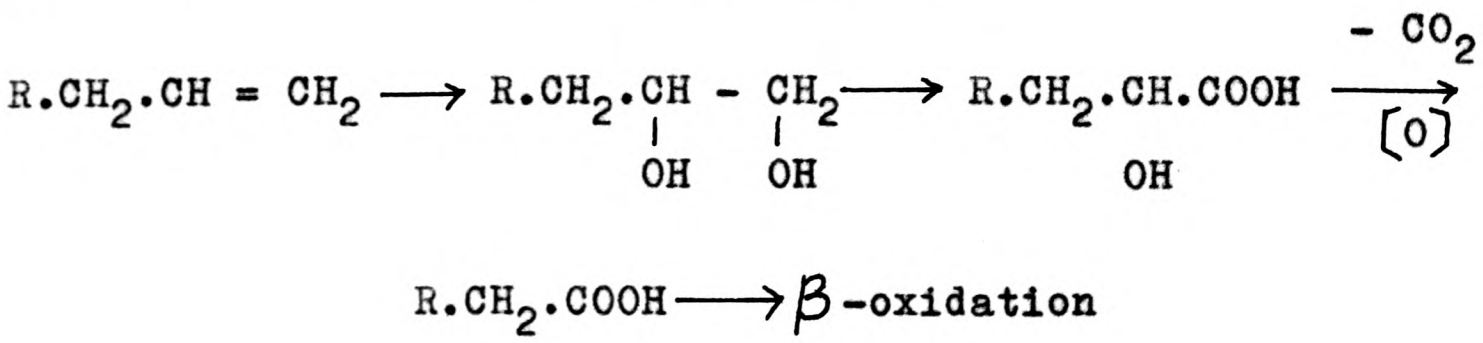


Figure 7 α -Olefin Oxidation



from higher hydrocarbons, but failed to accumulate in detectable quantities. The methyl ketone of n-C_{11} alkane was later positively identified (Lukins and Foster, 1963). Working with Ps. aeruginosa growing on n-decane, Fredricks (1967) detected a variety of ketones and secondary alcohols. However, Mycobacterium rhodochrous, similarly grown, formed only the usual products of terminal oxidation followed by β-oxidation. At present the importance of ketone production as a dissimilative pathway in n-alkane oxidation is unknown.

Lukins and Foster (1963) isolated acetol as an oxidation product of acetone, but they were unable to determine the source of the oxygen which had been added to the acetone. O_2^{18} enrichment experiments with acetone- and acetol-grown cells showed equal amounts of isotope incorporation into cell material, suggesting that O_2 was fixed at a stage after acetol formation. O^{18} incorporation into acetol produced from acetone was poor. The propanedial pathway suggested by Rudney (1954) may operate (Fig. 4).

Levine and Krampitz (1952), studying the oxidation of acetone by a corynebacter, also detected acetol as an intermediate, but their pre-adaption studies excluded propanediol as an intermediate. Instead they suggested the pathway shown in Fig. 5.

Lukins and Foster (1963) suggested that 2-butanone is similarly converted to a hydroxy methyl ketone by the addition of a hydroxyl group to the C_1 position.

The metabolism of higher methyl ketones was studied by Forney, Markovetz and Kallio (1967). 1-undecanol was detected in the medium of a pseudomonad growing on 2-tridecanone. They postulated the mechanism shown in Fig. 6.

More recent studies (Forney and Markovetz, 1968) have shown the presence of trace amounts of undecyl-acetate in these cultures. The authors suggest that this ester was the intermediate product between 2-tridecanone and 1-undecanol. However, it may be that this compound is the result of an esterification of 1-undecanol and acetic acid and is a side reaction (cf. Micrococcus cerificans of Stewart and Kallio, 1959).

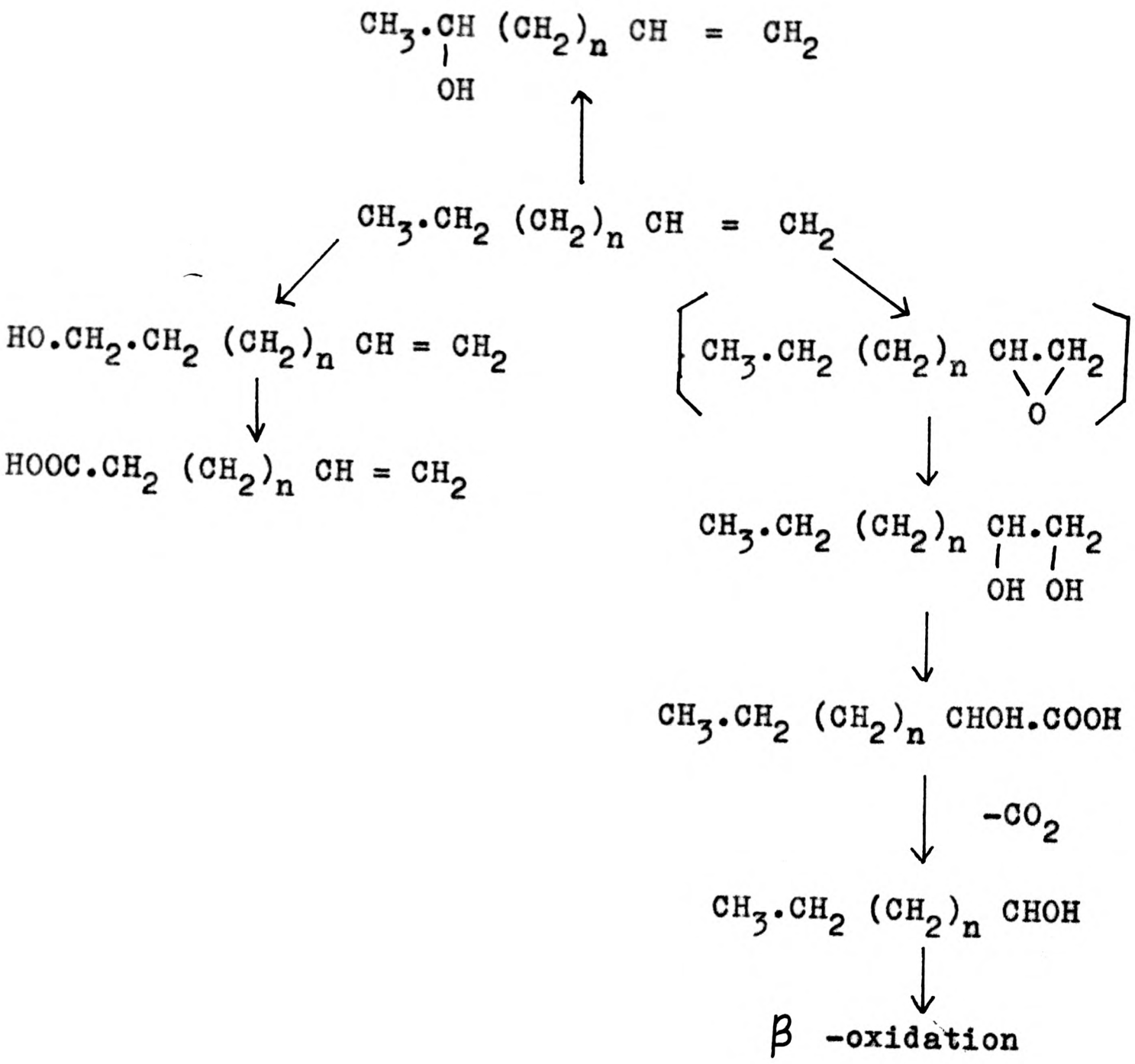
If such a compound was an intermediate in methyl ketone metabolism, it would seem that the degradation of long and short chain methyl ketones proceeded by two different pathways.

α-Olefin Metabolism

If a terminal desaturation process of the type described by Senez did operate, then the subsequent metabolism would be that of an α-olefin.

Ishikura and Foster (1961), when studying O_2^{18} incorporation with Candida lipolytica and an ethylene-utilizing bacterium, found 1-2 diols in the culture medium of the yeast growing on C_{16} and C_{18} α-olefins. Ethylene glycol was not detected in the case of the ethylene-grown bacterium, however there was O_2^{18} incorporation in the cells and the organism was

Figure 8 **Pathways of α -Olefin Metabolism**



known to grow on ethylene glycol. The authors suspected both oxygen atoms in the diols were derived from molecular oxygen. Foster (1962) proposed that this diol was further oxidised to form an α -hydroxy-acid which was decarboxylated and further degraded by β -oxidation (Fig. 7).

Huybregtse and van der Linden (1964), working with a pseudomonad, found α -olefin adapted cells had little tendency to attack the unsaturated end of the molecule and that no 1-2 epoxide (an intermediate in chemical oxidation of olefins) was formed from n-oct-1-ene. No primary saturated alcohols were detected as the products of β -oxidation proceeding from the unsaturated end of the molecule. They also suggested that a hydration mechanism, operating across the double bond, did not occur since n-octan-2-ol and 2-octanone were not detected.

It would seem that the tendency to attack the unsaturated end of an α -olefin depended upon the type of organiam. Klug and Markovetz (1968) working with C. lipolytica on 1-hexdecene and 1-heptadecene showed three modes of attack. The first involved methyl oxidation, producing ω -unsaturated alcohols, but the authors contended that this was not a major pathway. The second mechanism involved 1-2 diol formation, possibly with the 1-2 epoxide as an intermediate. Both of these oxidation products were detected, but only the diol would support growth. Finally, there was a sub-terminal oxidation at the saturated end of the molecule producing an ω -unsaturated secondary alcohol. The pathways are summarised in Fig. 8.

The readiness with which the unsaturated end of an α-olefin is attacked would seem to depend upon the organism. However, it is likely that most of the metabolic activity is directed to the ω-site, since no organism has been isolated capable of growth on an α-ω-diene (Huybregtse and van der Linden, 1964).

Hydrogen Autotrophy in Hydrocarbon Utilizing Bacteria

Tausz and Donath (1930) first reported this property in two ethane-utilizing bacteria. Similar observations were reported by Dworkin and Foster (1958) in four ethane-utilizing Mycobacterium sp. and a Gram-negative coccus. This property was also noted by Klausmeier, Brown, Benes and Strawinski (1958). In a detailed study of this feature, Lukins (1962) examined the possibility of a relationship between hydrocarbon utilization and hydrogen autotrophy, similar to that existing between nitrogen fixation and hydrogen autotrophy. However, the failure to detect hydrogenase activity in hydrocarbon-adapted cells, together with the inability of Hydrogenomonas sp. to utilize hydrocarbons, led him to conclude that no such relationship existed. It may be that these properties are merely coincidental to similar genera.

Microbial Utilization of Gaseous Hydrocarbons

The natural occurrence of gaseous n-paraffins has permitted the evolution of micro-organisms capable of exploiting these compounds as sources of carbon and energy. In following literature review, emphasis has been placed on ethane, propane and n-butane metabolism. Some references are made to methane and unsaturated gaseous hydrocarbons wherever pertinent. The utilization of methane, the first member of the series, is peculiar to a group of bacteria which may be regarded as strict methylotrophs, and is therefore not discussed.

The gaseous hydrocarbons in the environment appear to be derived from two sources: the immediate biosphere, or from petrochemical seepage. That biologically derived n-paraffins are products of microbial activity, was shown by Juranek (1959), who set up a series of enrichments to examine the evolution of gas from cellulose decomposition. Methane was produced in large amounts, 70% of the total, and ethane, propane and butane in amounts ranging from 100-500 p.p.m. Veber and Turkel'taub (1958) detected 0.1 - 0.18% of these latter gases in the gaseous phase above stored estuary mud sediments. Other biologically produced hydrocarbons are ethylene and propylene (Davis and Squires, 1954). Ethylene is a product of fungi and of plant tissues (Nickerson, 1948; Williamson, 1950; Young, Pratt and Biale, 1952). The ratio of other gaseous hydro-

carbons to methane in these fermentations is very low, of the order 1:1,000. Growth at these concentrations was reported by Rusakova (1960), when he cultivated an ethane-utilizing organism in sandy clay blocks in an atmosphere containing 30 p.p.m. ethane.

The ratio of methane to other hydrocarbons in soils where natural gas seepage occurs, is much higher, 10 or 20:1. This enhanced concentration has been used in microbial petroleum prospecting in the U.S.S.R. and the U.S.A. Originally, microbial surveying was based upon the discovery of regions containing numerous methane-oxidising micro-organisms. However, such features were not necessarily indicative of petroleum seepage, since biological methane production occurs commonly in soils. Greater importance in prospecting was therefore attached to the presence of ethane- and propane-utilizers. Davis, Raymond and Stanley (1959) advocated the detection of ethane oxidisers, whilst in the U.S.S.R. greater significance was attached to the occurrence of propane-oxidising micro-organisms, since ethane oxidisers tended to be uncommon, even in soils of petroleum-bearing regions (Bokova, 1954; Kartsev, Tabasaranskii, Subbota and Mogilevskii, 1959). Butane-oxidisers have been shown to be less specific than ethane-utilizing bacteria in prospecting surveys (Davis et al., 1959). Higher n-paraffins are of little use, since their physical properties do not facilitate seepage and in any case there is a wide variety of soil micro-organisms capable of utilizing these substrates (Foster, 1962;

Fuchs, 1961).

An investigation of the literature shows very few papers on the specific topic of gaseous hydrocarbon-utilizing micro-organisms. Much of the cited work is derived from literature of microbial petroleum surveying and is often merely a description of the colonial and microscopic appearance of the isolates, perhaps with a reference to their substrate spectra. Bokova (1954) described Mycobacterium perugosum var. ethanicum which utilized ethane and higher hydrocarbons, but not methane; and M. rubrum var. propanicum which grew on propane and higher hydrocarbons. Both organisms were capable of growth on orthodox organic media. Davis, Chase and Raymond (1956) isolated a variety of ethane-utilizing micro-organisms from soil in seepage areas. One, a new Mycobacterium species, grew on ethane and higher hydrocarbons only. They named this organism M. paraffinicum. Other acid-fast bacteria, non acid-fast Nocardia, Streptomycetes and a fungus were also isolated WITH ethane. These organisms could utilize a variety of organic media. Davis (1967) reported the discovery of other ethane-oxidising mycobacteria from similar sites. These differed from M. paraffinicum in colonial form and pigmentation and by growing on peptone agar.

The propane-using isolate of Kuznetsov and Telegina (1957) grew on organic substrates as well as n-paraffins. Despite the lack of hydrocarbon specificity, the presence of this organism in soils was taken as being indicative of petroleum

seepage, since the rates of propane utilization were found to be similar in the presence or absence of glucose, indicating hydrocarbons as preferential substrates.

Davis (1967) reviewed some of the findings of Russian microbial surveying of ground water. Telegina (1963) found that isolates from such sources lost their ability to grow on hydrocarbons after subculturing on non-hydrocarbon media. Slavnina (1963) was reported as finding that thermophyllic mycobacteria were more active in butane-oxidation than mesophyllic strains. In earlier surveys, Smirnova (1961) commented upon the rarity of ethane and propane utilizers in ground waters compared with methane oxidisers. In 1962, he found that nitrogenous petroleum compounds could serve as nitrogen sources for methane- and propane-utilizing pseudomonads and mycobacteria. NEW PARAGRAPH Telegina (1961) reported that some pseudomonad species were specific for methane alone, or for methane and higher hydrocarbons; whilst mycobacteria utilized hydrocarbons higher than methane. Prolonged subculture on non-hydrocarbon medium caused some isolates to lose their ability to grow on hydrocarbons. These latter findings differ from those of Kersten (1963), who found methane and propane isolates still capable of growth on these gases after two years of culture on peptone media. The methane-oxidising mycobacteria which he isolated, were reported as being Mycobacterium flavum var. methanicum, a species first described by Nechaeva (1949), when M. methanicum n. sp. was also isolated. Nechaeva

claimed that both could utilize propane, and that the former also grew on n-heptane.

With the exception of Davis and his associates, American research into gaseous hydrocarbon-utilizing micro-organisms was of an academic nature. Dworkin and Foster (1958) attempted an enrichment for methane utilizers using natural gas with ethane as an impurity. No methane oxidisers were isolated, but several ethane-utilizing bacteria and a fungus were obtained. This fungus was classified as a member of the family Moniliales and assigned to the genus Aeremonium. Of the bacteria isolated, five were Mycobacterium strains, one Gram-negative coccus, one Flavobacterium and one Alcaligenes. Two Mycobacterium isolates proved to be very similar to M. paraffinicum of Davis et al. (1956). Of the two hydrocarbons tested, n-hexadecane and ethylene, only the former supported growth. The remaining mycobacteria grew on a variety of non-hydrocarbon substrates, suggesting they were similar to M. perugosum var. ethanicum of Bokova (1954).

Kester (1961) isolated 12 different propane-oxidising bacteria and tested their substrate spectra with 50 hydrocarbons. Among his isolates were a Corynebacterium species and a Nocardia species which grew on 1-chloro-n-alkanes of carbon numbers C_3, C_4, C_5, C_6, C_8, C_{10} and C_{16}. None of the 2-chloro-alkanes tested or 1-bromo and 1-iodo propane were attacked. This Corynebacterium species was further examined by Kester and Foster (1963). Generally, better growth was

obtained with long chain hydrocarbons. When grown on C_{10} - C_{14} n-alkanes, this organism produced dioic-acids by diterminal oxidation.

Perry (1968), in substrate specificity studies, used this organism; a propane-utilizing Brevibacterium species, obtained from Foster; and a second Brevibacterium, isolated on 2-methyl-butane by Ooyama and Foster (1965). Of the gaseous hydrocarbons tested, Perry found that the Corynebacterium sp. would grow only on propane, but a non-proliferating suspension PREVIOUSLY grown on propane was capable of oxidising all gaseous n-alkanes except methane. The two Brevibacterium strains were reported as growing on C_1 - C_4 alkanes and a non-proliferating suspension grown on any gas was found capable of oxidising the other gases. Simultaneous adaption studies on C_1 - C_8 hydrocarbons, alcohols, fatty acids and methyl ketones were also reported.

One instance of nitrogen fixation has been reported by Coty (1967). From enrichments, he isolated a butane-utilizing Mycobacterium which also fixed nitrogen. The name M. butanitrificans n. sp. was proposed.

From the foregoing review of literature, it is apparent that little basic research has been performed upon the biology of gaseous hydrocarbon metabolism in comparison with the higher n-alkanes. Much of the Russian work on the substrate spectra of numerous isolates is very contradictory, particularly with isolates which appear to grow on methane and a plethora of hydrocarbon and organic substrates. As it is now thought that

methane-utilizing bacteria are obligate methylotrophs (Whittenbury, private communication), there are two possible explanations: either pure cultures were not used, or the gases themselves were contaminated with other gaseous alkanes which the organisms were using. Merely having a great preponderance of one gas, in no way guarantees that the organism will preferentially use it (Dworkin and Foster, 1958; Davis et al., 1956).

The range of organisms using ethane, propane and n-butane would seem, apart from a few exceptions, to be confined to the order Actinomycetales, the most frequently cited genera being Mycobacterium and Nocardia. There would seem to be no record of a yeast growing on short chain n-alkanes. The lowest member of the series utilized by a yeast was n-C_9 (Lowery, Foster and Jurthshuk, 1968). This reluctance to grow on short chain members may be due to damage to cellular membranes by these lipid solvents. However, this would not be the case with gaseous hydrocarbons. The tendency for bacteria to utilize gases has not been examined in great detail, since many substrate surveys did not include gaseous hydrocarbons, and very often the bacteria were isolated on higher paraffins themselves. Lukins (1962) found a preference towards liquid hydrocarbons. Of 21 strains of a Mycobacterium species, he found only a few which grew on gases. The findings of Tausz and Donath (1930) of a n-hexane-isolate growing on all gaseous n-alkanes may be exceptional.

Studies on the pathway(s) of gaseous hydrocarbon metabolism appear to be contradictory concerning the participation of unsaturated intermediates. Klausmeier *et al*. (1958) and Davis *et al*. (1956), merely on the basis of substrate utilization and simultaneous adaption experiments, respectively suggested that propylene and ethylene were intermediates in propane and ethane metabolism. However, the co-oxidation, deuterium experiments of Leadbetter and Foster (1960) and Lukins and Foster (1963) excluded these olefins as possible intermediates. Thus it would seem that ethane is oxidised via ethanol, acetaldehyde to acetic acid. Propane and butane appear to be oxidised either by a pathway involving the primary alcohol to the acid; or by a methyl ketone intermediate, the second step involving the hydroxylation of the adjacent methyl group. It may well be that both of these routes are used simultaneously, if the alcohol and methyl ketone are formed by an oxidation involving a free radical mechanism.

Lipids Formed by n-Alkane-grown Bacteria

Since the first products of the metabolism of paraffins appear to be primary alcohols and fatty acids of the same carbon number AS THE SUBSTRATE, it is not surprising that the chain length of the n-alkane greatly influences the types and amounts of lipid materials synthesised by the cell.

Stewart et al. (1959) isolated a Gram-negative coccus (from n-C_{16} enrichments) which accumulated waxes. This property was exploited in a series of experiments which indicated that the early oxidation products were primary alcohols and fatty acids. Preliminary investigations showed that this organism produced cetyl palmitate when grown on n-C_{16}. In a later experiment, Stewart and Kallio (1959) grew this organism on a variety of hydrocarbons, n-C_{12}, C_{14}, C_{16} and C_{18}. Generally the acid moieties of the esters produced contained 16 carbon atoms, and the alcohol was of the same chain length as the substrate. With n-C_{18}-grown cells, a second wax was produced which had 18 carbon atoms in its acid moiety. The alcohol in the wax of n-C_{12}-grown cells was unidentified. This unusual organism showed its preference towards oxidation of the methyl end of an α-olefin, by producing unsaturated esters from tetradec-1-ene (Stewart et al., 1960). The classification studies of Finnerty, Hawtrey and Kallio (1962), involving several strains, assigned them all to the genus Micrococcus suggesting a new species name,

Table 1

Strain	Lipid % dry weight
Brevibacterium sp.	9.1
Nocardia sp.	5.8
Mycobacterium sp.	3.9

Total chloroform-methanol extractable lipid of propane-grown cells

Table 2

Analysis of Hydrocarbon-grown *Candida* Cells

	Lavera	Grangemouth
Moisture % by wt.	5	5
Nitrogen "	10.6	10.0
Crude protein % by wt.	66	62
Total lipids "	0.5	8
Pepsin digestibility %	83	83

M. cerificans. Stevenson, Finnerty and Kallio (1962) examined ester production from odd numbered n-alkanes from C_{11} to C_{17}. Although all supported growth, waxes were produced from n-heptadecane only. Three waxes were formed, and as before the alcohol was of the same carbon number as the substrate. The acids contained 15, 16 and 17 carbon atoms. The authors suggested that pentadecanoic acid was a product of β -oxidation of heptadecanoic acid, and that hexadecanoic acid was formed by α -oxidation of heptadecanoic acid rather than by de novo synthesis. Makula and Finnerty (1968), in similar work, demonstrated that the fatty acids of M. cerificans grown on non-hydrocarbon substrates and even carbon numbered n-alkanes from C_{10} to C_{18} contained even numbers of carbon atoms in both cases. Those extracted from odd n-C_{11} - n-C_{17} alkane-grown cells contained both odd and even numbers of carbon atoms, with a greater tendency for the odd numbered acids to become predominant with increasing n-alkane carbon number.

Studies on the triglyceride fraction of the lipid of three strains (a Brevibacterium, a Nocardia and a Mycobacterium) were performed by Dunlap and Perry (1967) to examine the fatty acid variation with different substrates. Acetate-grown cells had C_{16}, $C_{16:1}$, C_{18} and branched C_{19} as their predominant fatty acids, whereas propane-grown cells had C_{15}, $C_{17:1}$, C_{18} and branched C_{19}. The latter finding would suggest a 2 carbon addition to a 3 carbon fragment as a synthesis mechanism. The Mycobacterium when grown on C_{13} - C_{17} n-alkanes had a major fatty acid component of the same carbon number. (See Table 1

for the dry weight percentages of the lipid fractions of the strains grown on propane.) Further examination of this feature (Dunlap and Perry, 1968) showed that n-alkanes of carbon number less than 13 and greater than 17 were not incorporated directly, but only after degradation, since cells grown on these substrates had fatty acid profiles similar to acetate-grown cells. C_{14} to C_{17} 1-alkenes were incorporated as ω-monoenoic fatty acids. Methyl ketones had 1 or 2 carbon atoms removed from their skeletons before incorporation. Ketones, other than methyl ketones, were degraded and fatty acid synthesis proceeded anew.

Edmonds and Cooney (1969) growing Pseudomonas aeruginosa on n-C_{13}, a jet fuel hydrocarbon mixture and tripticase soy broth, found that the total lipid in each case was 7-8% of the dry weight. With organisms grown on tridecane, there was little fatty acid of this chain length present, which led the authors to suggest that synthesis was from scratch by 2 carbon fragments.

The investigations of Raymond and Davis (1960), with an ethane-isolated Nocardia strain, examined in detail the cellular components and yields after growth on various n-alkanes under different environmental conditions. The lipid content of cells grown on glucose, n-C_6 and n-C_{13} was 26-28% of the dry weight. No waxes were formed, but n-C_{16} and n-C_{18}-grown cells had 48 and 56% respectively, of which 38-39% was cellular waxes. The authors suggested that these waxes represented the esterification products of an accumulation of n-alkane oxidation products,

but they were unable to attribute any function to them other than storage products. Davis (1964a) made a more detailed survey of the triglyceride and ester fractions of these lipids. He found that the predominant fatty acid in the triglyceride fraction of n-C_{13} to n-C_{19}-grown cells had the same carbon number as the substrate. The same was also true of the fatty acid of waxes of n-C_{16} to n-C_{19}-grown cells and alcohol moiety was always of the same carbon number. In a later paper (Davis, 1964b), the absence of wax accumulation with short chain alkanes was again exhibited when propane and n-butane were used as substrates. Instead, these cells accumulated poly β -hydroxybutyric acid (P.H.B.) amounting to 4% of the dry weight in C_3-grown cells, and 12-14% in C_4-grown cells. Infrared spectra of this polymer in butane-grown cells suggested that this lipid existed as a co-polymer of β -hydroxybutyric and β -hydroxybutenoic acids. The fatty acid profiles of these gaseous hydrocarbon-grown cells were almost identical, containing mainly palmitic and stearic acids. The total lipid content of C_4-grown cells was 45 to 55% of the dry weight.

Lipids of Hydrocarbon Grown Yeasts

Mizuro, Shimojima, Iguchi, Takeda and Senoh (1966) analysed the fatty acids of Candida petrophillum and Torulopsis petrophillum grown on n-C_{13}-alkane and found that the odd and even fatty acids were present in equal amounts. When grown on n-C_{13}, n-C_{16} and glucose, half of the lipid content of the

Candida sp. was phosphatidyl ethanolamine and phosphatidyl choline. No free alcohols or waxes were detected.

SUMMARY

Generally it would seem that when the carbon number of the n-alkane is above 12 or 13 and below 17 or 19 the triglyceride formed by the micro-organism tended to consist of fatty acids of the same carbon number. The same was true of waxes formed, although the acid moiety usually had a carbon number of 16. The direct incorporation of n-alkane oxidation products does not appear to happen with n-paraffins of carbon number outside the range described above. Instead, lipid synthesis is de novo by 2 carbon addition. When propane and butane are used as substrates no waxes are formed, but poly-β-hydroxybutyrate accumulates, particularly with butane.

Hydrocarbon Derived Biomass

The potential of unicellular biomass as a source of protein for animal and human nutrition has long been realised. For general reviews see Bunker's chapter in "Biochemistry of Industrial Micro-organisms" (Rainbow and Rose, 1963), the chapter headed "Mass Cultivation of Micro-organisms" in "Industrial Microbiology" (Rose, 1961) and Mateles and Tannenbaum (1968).

Previously only carbohydrates had been considered as carbon and energy sources for biomass production, but Davis (1967) reported the studies of Just and Schnabel (1948) which involved the growth of bacteria on paraffins giving 100% yields on a dry weight basis. Later work by Just, Schnabel and Ullmann (1951) reported high yields of paraffin-grown cells of a Candida species. From the results of experiments with paraffin-grown C. tropicalis, Hoerburger (1955) concluded that protein production from hydrocarbon material was not economically feasible, as too high an aeration rate was necessary.

Detailed studies of protein and lipid content, and cell yields of hydrocarbon-grown organisms were performed by Raymond and Davis (1960) and Raymond (1961) using Nocardia and Candida species. Raymond (1961) suggested that the rapid assimilation and high rate of conversion of substrate into cellular material made n-alkanes attractive sources for industrial microbial processes.

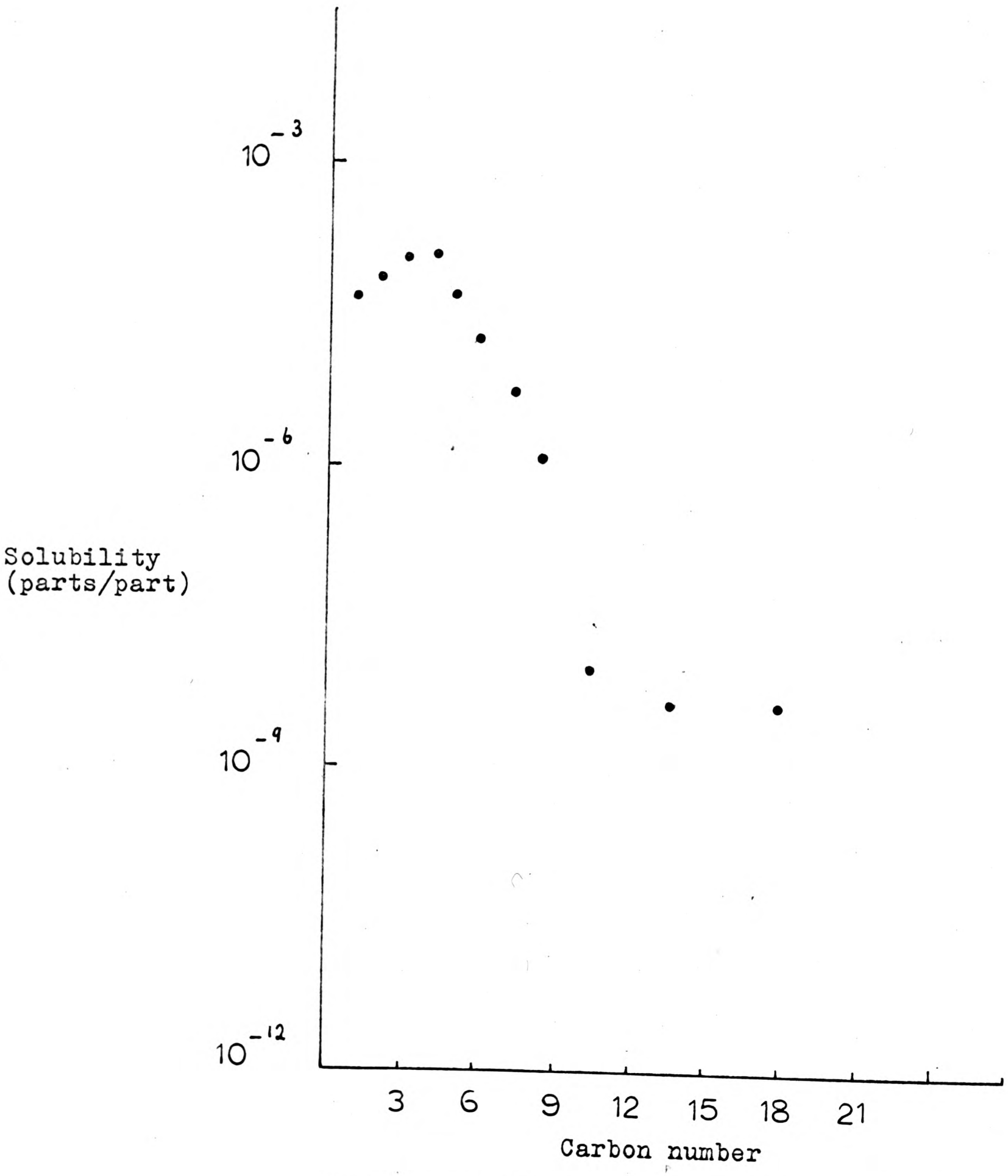
The first serious studies on the exploitation of petroleum compounds by micro-organisms were those of Champagnat, Vernet, Laine and Filosa (1963a, b) at the British Petroleum Refinery at Lavera in France. It was suggested that micro-organisms could be used to dewax gas oil by selectively metabolising the n-paraffins. Such a process, if extended to a large scale continuous culture plant, would also yield large amounts of biomass. The separation of the organism, a yeast, from the residual gas oil would complicate the process, since a lipid solvent would be necessary to remove traces of gas oil, toxic to animals. This problem has been circumvented by growing the yeast on pure n-paraffins, which are completely metabolised. Such a plant has been set up by B.P. at Grangemouth, Scotland, where there is an abundant source of these paraffins. There would seem to be little difference between the cell material derived from these two processes (Table 2). It was found necessary to supplement the yeast fodder with the essential amino acid methionine, as the concentration in the yeast protein was too low.

Further studies on the suitabilities of n-alkanes as substrates were performed in the U.S.A. by Johnson and his associates. Miller, Lie and Johnson (1964) grew a soil isolate, resembling Candida intermedia, on n-alkanes from C_{12} to C_{18}. They noted that the growth rate increased with substrate chain length, the shortest generation time being 4.5 hours with octadecane. Yields of 82% were obtained with

even carbon-numbered n-alkanes from n-C_{14} to n-C_{18}, but yields decreased with chain lengths of C_{12} and less, and also with odd carbon-numbered n-paraffins. The nitrogen content of cells ranged from 6.9 to 7.5%. Organisms grown on n-heptane had the highest lipid content, 10.3%. Similar low CELL yields were obtained by Arima, Ogino, Yano and Tamura (1965) when a Pitchia species was grown on n-C_{10} to n-C_{13} alkanes.

In a second paper, Miller and Johnson (1966a) reported the results obtained by growing a mixed culture of C. intermedia and C. lipolytica. (Presumably the former species supplied some growth factor(s), since the latter would not grow alone in unsupplemented salts medium.) Higher n-paraffins were used in this work, ranging from n-C_{15} to n-C_{28}. Those which were solids were dissolved in a biologically-inert branched hydrocarbon, PRISTANE. Cell yields of 74-90% and generation times of 3-8 hours were obtained. The fastest growth rates and highest yields were obtained on n-C_{22}. Analysis of cells showed crude protein yields of 34-48% and lipid contents of 2-13%. This same mixed culture was used in a second experiment in which the above characteristics were measured during utilization of n-alkanes in a gas-oil fraction (Miller and Johnson, 1966b). Generation times of 4-9 hours were reported, and cell yields of 70-90%, based on n-paraffin utilization, were achieved. The cells most readily removed paraffins in the n-C_{14} to n-C_{24} range. Nitrogen determinations showed a content of 8.8 - 9.3% of the dry weight.

Figure 9



Water solubility of n-paraffin at 25°

Attractive as these figures of yield, generation time and crude protein content may seem, several drawbacks to yeast biomass production exist (Humphrey, 1967). The biological requirement for oxygen is about three times greater when cells are growing on such a reduced energy source as hydrocarbon, than when carbohydrate is supplied. Since most industrial fermentations are oxygen limited, it would seem that the production rate would be reduced when a hydrocarbon substrate is used; however, this would be financially compensated for by a higher yield factor, 100% with hydrocarbon, compared to 50% with carbohydrate (Darlington, 1964; Guenther, 1965). Humphrey also drew attention to the insolubility of these liquid paraffins (Fig. 9) and the problems of removing unused gas oil from the yeast cells.

These last two factors could be overcome by using gaseous hydrocarbons, since there would be no residual substrate attached to cells, and they are 10^4 times more soluble in water (Fig. 9). Nevertheless, work with such gaseous substrates poses problems of explosiveness and the possible necessity of recycling the gas atmosphere in the fermentor. These problems are discussed by Hamer, Heden and Corenberg (1967) who worked with a recirculating methane/air mixture.

At present there is greater interest in methane as a substrate than the other gaseous n-alkanes, primarily because of its abundance and cheapness. Several petroleum and industrial concerns are, to varying degrees, interested in methane

fermentation, but an air of secrecy surrounds results, particularly those of protein content, yield and generation time.

Coty (1967) reported a nitrogen-fixing, methane-utilizing Pseudomonas which had a protein content of 46-48% and contained 25% lipid, of which 72% was poly-β-hydroxybutyrate. Growth rates were not reported. Wolnak, Andreen, Chisholm and Laadeh (1967) were sceptical about biomass production from methane, since the protein content of cells was low, 32-36%, but they suggested vitamin or specific amino acid production might be an economically viable concern. In a description of numerous methane-utilizing isolates, Whittenbury (1969) reported some as having a generation time of 4-5 hours. Crude protein content varied between 35 and 70% of the dry weight. These results were obtained from batch culture, and the cells' behaviour in continuous culture awaits elucidation.

The industrial potential of micro-organisms utilizing ethane, propane and butane has been neglected. Some work has been performed on the mechanism of metabolism of these substrates and some applied research into the use of such organisms in microbiological petroleum prospecting. Little information exists in the feasibility of using these hydrocarbons for biomass production. Davis (1964b) reported upon the lipid contents of C_3- and C_4-grown cells (see section on lipids). Perry and his co-workers are about to publish some data which may be applicable to biomass production.

It would appear that the production of biomass from hydrocarbon is economically feasible and that the protein formed is biologically acceptable (Johnson, 1967; Mateles and Tannenbaum, 1968). This is certainly so in the case of yeasts grown on liquid n-paraffins. At present, research in the utilization of gaseous hydrocarbons is in its infancy. Several problems, however, are immediately apparent. These substrates are only slightly soluble in water, and their flammable nature poses problems of fermentor design. The main drawback is perhaps the fact that only bacteria have been found to utilize these gases. All organisms proven to be methane utilizing are Gram-negative, which MAY raise problems of digestibility and toxicity. Organisms capable of utilizing the other gaseous hydrocarbons tend to be mainly acid-fast and have a high lipid content when grown on these substrates (Davis, 1964b).

S E C T I O N 1

Studies on Liquid Hydrocarbon Utilization by Yeasts

Table 3

Analysis of Liquid Hydrocarbon Fraction

		Percentage weight
Linear	C_{10}	-
	C_{11}	1.5
	C_{12}	3.0
	C_{13}	6.7
	C_{14}	15.3
	C_{15}	25.5
	C_{16}	26.4
	C_{17}	16.3
	C_{18}	3.1
	C_{19}	0.2
	C_{20}	-
	Total	98.8

MATERIALS AND METHODS

Growth Conditions

Unless otherwise stated, incubation was at 30°. Except in the initial stages of enrichment and isolation, liquid cultures were incubated in a Gallenkamp Orbital Shaker, operating at 250-300 r.p.m.

Storage of Materials

Stock cultures, media and samples of mud for isolation studies were stored at 4°.

Sterilisation

Unless otherwise stated, all materials were autoclaved at 121° for 15 minutes.

Special Materials

Pristane (2,6,10,14-tetramethylpentadecane) was obtained from Kock-Light Laboratories.

The analysis of the n-alkane liquid hydrocarbon used as feedstock in this investigation is shown in Table 3. This fraction and its analysis were obtained from British Petroleum Ltd., Grangemouth.

Enrichment Medium

The basal salts solution was prepared as follows:

KH_2PO_4, 2.0 g.; $MgCl_2.6H_2O$, 0.1 g.; NH_4SO_4, 1.0 g.; $MnCl_2.4H_2O$, 0.1 g.; Sequestrene-iron complex C.P. 2 Geigy (Johnsons of Hendon Ltd.), 0.1 g.; trace elements solution, 0.1 ml. were dissolved in 1 l. distilled water. The pH was adjusted by addition of 1N - H_2SO_4 or 1N - NaOH solution to 4.0 or 6.0.

The trace elements solution contained $AlCl_3$, 1.0 g.; KI, 0.5 g.; KBr, 0.5 g.; LiCl, 0.5 g.; $MnCl_2.4H_2O$, 7.0 g.; H_3BO_3, 11.0 g.; $ZnCl_2$, 1.0 g.; $CuCl_2$, 1.0 g.; $NiCl_2$, 1.0 g.; $CoCl_2$, 1.0 g.; $SnCl_2.2H_2O$, 0.5 g.; $BaCl_2$, 0.5 g.; Na_2MoO_4, 0.5 g.; $NaVO_3$, 0.1 g.; Se-salt, 0.5 g. Each salt was dissolved separately in distilled water and the pH value adjusted to 6.8 - 6.9 before mixing. The final volume of the complete mixture was 18 l. and the pH value 3.0 - 4.0. (PFENNIG) PR

Solid medium was prepared by adding No. 2 Ion Agar to a concentration of 1.5% w/v. Liquid hydrocarbon (5.0% v/v) was added to the salts solution before sterilisation. When agar medium was used in a Petri dish, the hydrocarbon was supplied in the vapour phase by adding a few drops to a piece of filter paper adhering to the inside of the lid.

Enrichment Procedure

Samples of mud and soil were collected from areas likely to be contaminated by industrial effluent containing hydrocarbons. Samples (1.0 ml.) were added to 10 ml. of medium in 25 ml. flasks, and 5 drops of hydrocarbon were added to each.

These enrichments were incubated for 2 days in a shaking water bath. Samples (1.0 ml.) were then removed and subcultured in similar flasks for 3 more days. This procedure was performed in duplicate for each sample with one series of enrichments at pH 4.0 and the other at pH 6.0.

After subculture, samples were plated out on hydrocarbon medium at pH 6.0. Two to 5 days later, numerous colonies were visible. Those containing yeast cells were further plated out until pure cultures were obtained. Stock cultures of these isolates were stored on Oxoid Sabouraud Dextrose Agar.

In an attempt to reduce the growth of non-hydrocarbon utilizing micro-organisms during enrichment, a small inoculum, 1.0 ml., of the sample was taken to minimise carry-over of extraneous organic materials. The use of media at pH 4.0 and 6.0 to select yeasts possessing different pH optima was later shown to be an unnecessary procedure, since the isolates were found to have an optimum range for growth to proceed between pH 5.0 and 6.0. This technique proved suitable for the isolation of hydrocarbon-utilizing yeasts and bacteria.

Growth Measurement Techniques

The physical properties of the hydrocarbon in an aqueous medium rendered orthodox techniques for measuring growth impossible, since the hydrocarbon formed a droplet suspension. As a result, the following techniques were developed to solve this problem.

In experiments where it was necessary to determine the dry weight of cells per ml. of sample, the following procedure was adopted. The growth vessel was givorously agitated to give a homogeneous suspension of droplets and cells. A sample was rapidly withdrawn before the emulsion separated, and pipetted into a 10 ml. disposable syringe barrel, to which was attached a Millipore filter cassette. The membrane inside was of known weight, 13 mm. in diameter with a pore size of 1.2μ. Usually a 10 ml. sample was taken for filtration, but if growth was dense, a 5 or 3 ml. sample was withdrawn. During the latter stages of filtration, when a large mass of cells on the membrane impeded the flow, it was frequently necessary to place the syringe assembly in a bench vice to bring additional pressure to bear on the plunger. After filtration, the excess moisture was removed by placing the membrane on filter paper for a few seconds. The membrane and attached cells were dried for 2 days at 104^{o} to remove all remaining hydrocarbon. An Oertling single pan balance was used to weigh to 0.1 mg. Each dry weight determination was performed in duplicate and the mean taken.

When it was necessary to detect only large differences in growth, the following method of comparative measurement was employed using an inoculated hydrocarbon-free control. Growth was observed visually, either <u>in situ</u> in the culture vessel, or, if the optical density was very great, by diluting samples in a test tube. In both cases the sample was allowed to stand

until all but the smallest hydrocarbon droplets had risen to the surface, leaving behind a suspension of cells, the turbidity of which was indicative of the total growth.

Preparation of Inocula

Unless otherwise stated, inocula were always taken from stock cultures. The yeast strains were streaked out on to plates of Sabouraud agar and incubated overnight. Adaption to hydrocarbon was effected by subculturing into liquid culture containing hydrocarbon and growing for 1 day. Hydrocarbon-free inocula were obtained by allowing the growth flask to stand for 10 minutes, before withdrawing a sample from the aqueous phase.

Medium Modifications

1) pH variation. Eight isolates were chosen for this test, 3 pigmented strains and 5 of the 25 non-pigmented strains. The pH range tested was from 3.0 to 6.0, the actual pH values differed from each other by 0.5 of a unit. The pH was adjusted by the addition of NaOH and H_2SO_4. The isolates were adapted to hydrocarbon in a liquid medium of pH 5.0. Five ml. of inocula were added to 200 ml. of the different media and the pH value recorded. Growth was measured visually after 2 and 4 days, after which time the change in pH of the medium was noted. Readings at 2 days gave a better indication of the pH optima than those on the fourth day, which merely

displayed the total pH range over which growth was possible.

2) Trace elements concentration. Trace elements solution concentrations of 0.05, 0.025 and 0 ml. per l. of salts solution (pH 5.5) were examined for their effect on growth of 8 strains thought to be representative of the isolates. Growth was measured visually after 2 and 4 days.

3) Yeast extract supplementation. After adjustment of H^+ and trace element solution in the basal salts solution, the medium was supplemented with 0.01% (w/v) Oxoid Yeast Extract. The growth of 8 representative isolates was observed in this medium when compared with controls containing either no hydrocarbon, or no yeast extract. Results were read after 2 days.

4) Growth factor supplementation. The basal salts solution was supplemented by the addition of the following growth factors, thiamine, pyridoxine, riboflavin, biotin, cobalamine, calcium pantothenate, nicotinic acid, folic acid, ascorbic acid and inositol. Stock solutions were prepared by dissolving these growth factors in distilled water at a concentration of 1 mg. per ml. The one exception was folic acid, which was not completely dissolved at this concentration and was sterilised with undissolved crystals still present. Sterilisation was effected by Seitz filtration.

Each growth factor was tested at 2 concentrations in the medium, 1×10^{-2} and 10^{-3} mg. per ml. In addition to testing each separately, the possibility of a multiple requirement was examined. One non-pigmented isolate was chosen as the test

organism. Inoculated controls containing yeast extract and basal salts solution were alsoset up. Growth was measured visually after 2 days.

Classification Techniques

The 28 isolates (3 pigmented and 25 non-pigmented) were classified according to Lodder and Kreger van Rij (1952).

1) Morphology. Cellular morphology and mode of vegetative reproduction was observed in cells grown on Sabouraud agar. Wet mounts were examined by phase contrast microscopy. Measurements were made with a stage micrometer.

2) Ascospore formation. Isolates were grown on Gorodkowa agar at 25° and examined microscopically over a period of 3 weeks for asci formation.

3) Ballistospore formation. Potato agar was used to promote ballistospore formation. Three isolates per dish were streaked on to potato agar in a Petri dish, the lid of which was then removed, and the base of a second dish containing Sabouraud agar attached. Preparations were incubated at 25° with the inoculated base uppermost. The Sabouraud medium was examined over 4 weeks for mirror-image growth. At the end of this period the preparations were opened and the growth on potato medium examined for ballisto-spores.

4) Colonial morphology. Morphology was observed on Sabouraud agar plates incubated at room temperature.

5) Pseudomycelium formation. This test was performed using slide cultures with potato agar. Preparations were examined over a period of 2 weeks.

6) Pellicle formation. During fermentation studies, the production of pellicles in the test tubes was noted.

7) Fermentation studies. The ability to ferment and produce acid from various sugars was tested. The sugars used were the d-isomers of glucose, galactose, sucrose, maltose, lactose and raffinose. They were added to give a final concentration of 2% (w/v) in the modified salts solution containing yeast extract, and dispensed into 6 by $\frac{5}{8}$ in. test tubes. A control tube containing no sugar was set up for each isolate. A saline (0·85% w/v) suspension of cells was used as an inoculum. Cultures were examined daily for a week. Those showing growth were tested for acid production by adding 3 drops of methyl-red indicator. (This reagent was prepared by dissolving 1 g. in 600 ml. of methylated spirits and diluting with 600 ml. distilled water.) A positive result was one in which a red colouration was formed. After 7 days the remaining tubes were tested.

8) Utilization studies. The isolates' ability to grow on various sugars without acid production was examined using the same series of tubes as were used in fermentation studies. A positive result was one where there was an improvement in growth over the control, but where the indicator did not turn red.

9) Nitrate utilization. The ability of the isolates to use nitrate as their nitrogen source was studied as follows. 0.2 ml. of a saline suspension was spread over the surface of N-free basal salts agar containing glucose (1.0% w/v). A drop of KNO_3 (1% w/v) solution was placed on the agar surface and the preparations were incubated for 2 days. A positive result was one in which there was enhanced growth in the region to which the KNO_3 had been added.

10) Ethanol utilization. Apart from using modified salts solution containing yeast extract, and incubating at 30°, this test was performed as described by Lodder and Kreger van Rij (1952). After 3 weeks a positive result was one in which there was more growth than in the ethanol-free control.

11) Arbutin splitting. The enzymic splitting of hydroquinone-β-D-glucoside was tested as follows. A solution of $FeCl_3$ (5% w/v) was filter sterilised and 1 drop placed in a Petri dish. A solution of arbutin (0.5% w/v) in glucose/salts agar was poured into the dish and mixed. Several isolates were streaked on to each plate and incubated for 6 days. A brown zone around the regions of growth indicated a positive result.

Substrate Availability Studies

Utilization of Dissolved Hydrocarbon

Ten ml. of hydrocarbon were shaken with 200 ml. salts solution in a separating funnel for 5 minutes. After the

emulsion had separated into 2 phases, the lower aqueous phase was withdrawn, sterilised and inoculated with 1 ml. adapted, hydrocarbon-free yeast suspension. A control flask, containing medium which had not been in contact with hydrocarbon, was also set up. Growth in both flasks was observed visually over 5 days.

Dialysis Experiment

Five ml. of hydrocarbon were enclosed in Visking 32/32 dialysis tubing and placed in a flask containing 100 ml. salts solution and sterilised. The vessel was inoculated as above. Growth was examined microscopically after 2 days.

Effect of differing Volumes of Substrate

Two flasks containing 200 ml. of salts solution were set up. Ten ml. of hydrocarbon was added to one, and 1 ml. to the other. Both were inoculated with 5 ml. of hydrocarbon-free, adapted cell suspension. The amounts of growth were visually compared after 1 day when unused substrate was still present.

Slide Culture

A glass slide was sterilised by dipping into ethyl alcohol and flaming. Molten agar medium, containing a suspension of hydrocarbon droplets, was run down one side of the slide and allowed to solidify in a sterile Petri dish before inoculation. A loopful of culture was carefully spread over the agar. No

coverslip was used in the preparation, since it tended to distort the droplets and excluded oxygen from the preparation. A distinctive field was chosen to help recognition when the slide was periodically observed. It was found necessary to incubate the preparation in a Petri dish containing moistened filter paper to prevent drying. Observations were made over a period of 5 to 10 days and a photographic record kept. Photomicrographs were taken with a Leitz Orthomax camera and microscope, using phase contrast.

Effects of Substrate Availability on Growth Rates

A series of solutions of hydrocarbon were prepared by dissolving the n-paraffin fraction in different volumes of pristane: solution A 83% (v/v) n-paraffins; solution B 17% (v/v) n-paraffins; solution C 100% (v/v) n-paraffins and solution D 0% (v/v) n-paraffins (i.e. pure pristane). Six ml. of each hydrocarbon mixture were separately added to flasks containing 200 ml. salts solution and inoculated with 5 ml. of an adapted hydrocarbon-free cell suspension. Samples were withdrawn daily for 4 days and the dry weight/ml. determined by filtration methods.

The tendency of the different hydrocarbon solutions to emulsify was measured as follows. Three ml. samples of the hydrocarbon mixtures were placed in 300 ml. of salts solution in Osteriser jars and blended for 1 minute in an Imperial VIII Osteriser at maximum speed. The emulsions formed were diluted

DOWN-DRAUGHT TUBE WITH BAFFLES FOR 500 ml. FERMENTOR

SCALE: 1/2

MATERIAL: STAINLESS STEEL F.D.P. QUALITY

FINISH: POLISHED

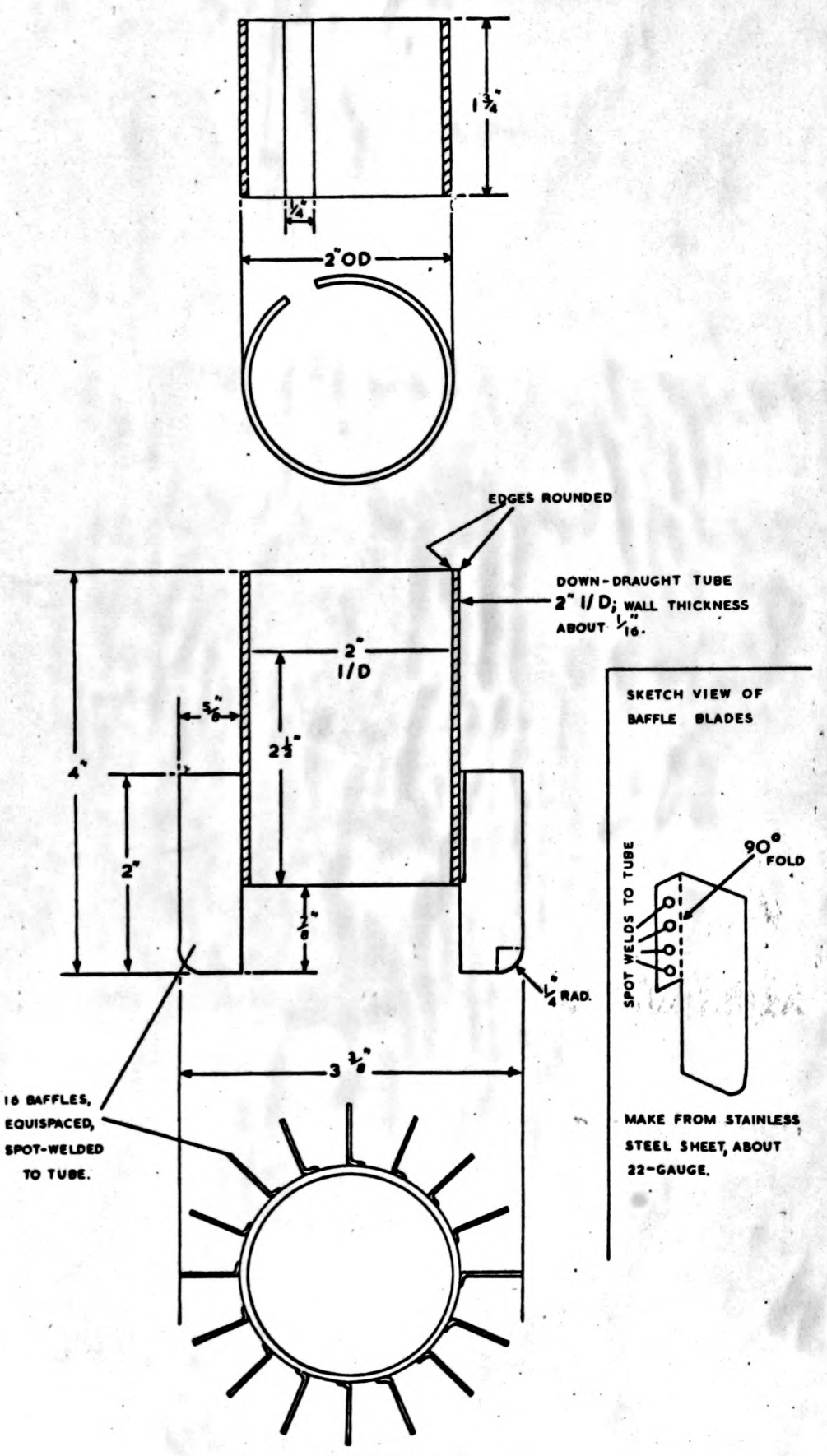
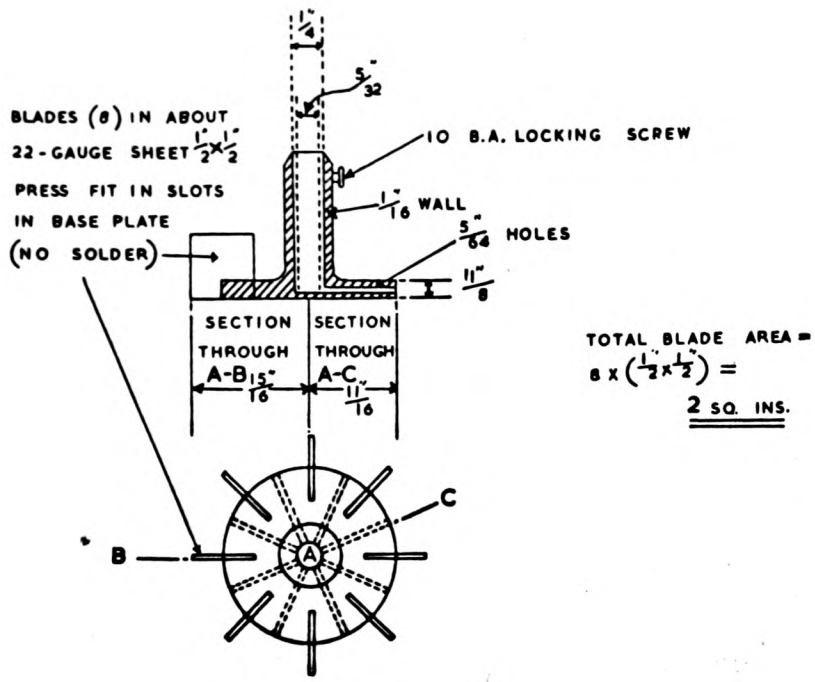


Figure 10

STIRRER FOR 500ml. FERMENTOR



TOTAL BLADE AREA = $8 \times (\frac{1}{2} \times \frac{1}{2}) =$ 2 SQ. INS.

PROPELLOR IN STAINLESS STEEL

SCALE 1/1

Figure 12

GLAND AND PROPELLOR SHAFT FOR 500ml. FERMENTOR

ASSEMBLY SCALE 1/1

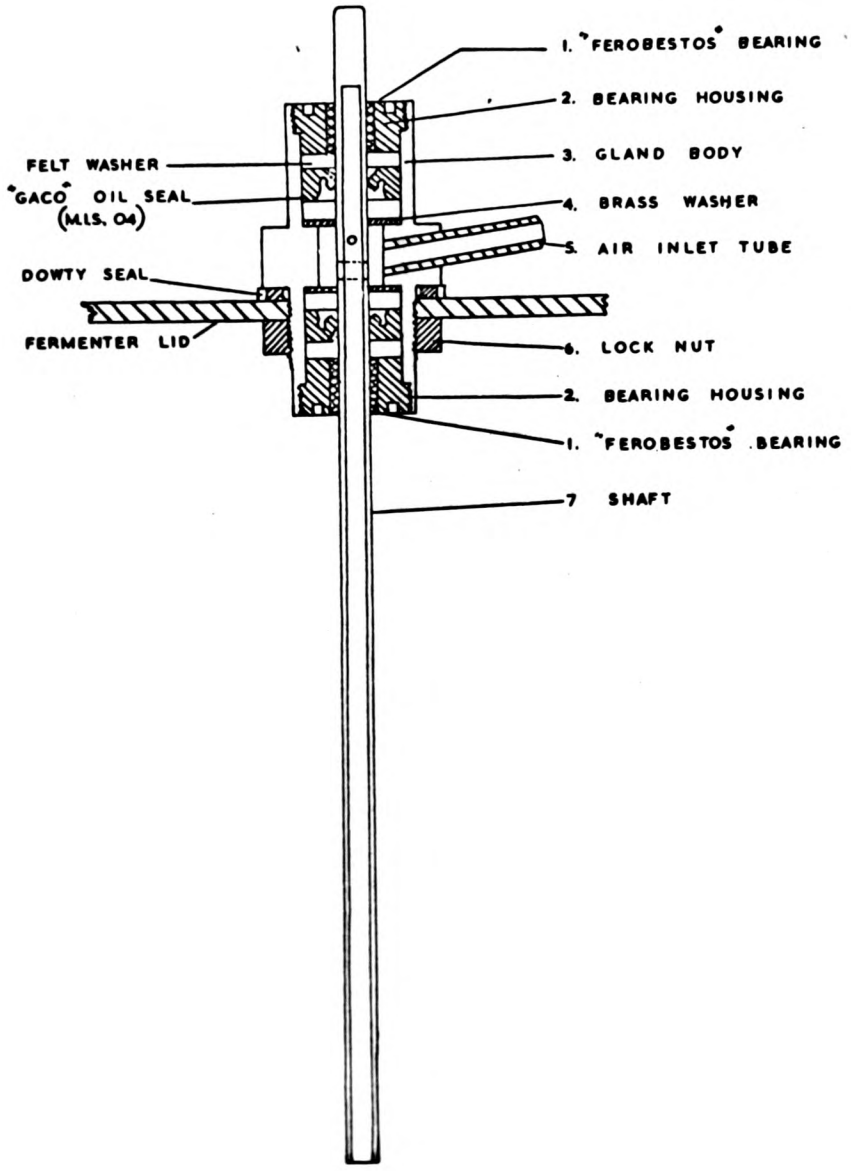


Figure 11

by a factor of 10 with fresh medium and the optical densities were measured at 540 nm. on an Unicam 500 Spectrophotometer. A glass cell of 1 cm. light path was used.

Continuous Culture Studies

Description of Apparatus

The type of chemostat used was a single stage vessel of 500 ml. working volume. The system incorporated the usual ancillary services of pH and temperature measurement and control, and variable air flow meters. Addition of aqueous medium was by a continuously variable-speed peristaltic pump. Hydrocarbon addition was by means of a Sage Syringe Micropump, which also incorporated a continuous range of speeds. The antifoam system was not used in these studies. For a more detailed description see Munro (1964).

Aeration and Agitation System

A description of the impellor, aeration and baffling systems within the vessel is given below since the effect of these features concern the observations in this report.

"The down-draught tube is shown in Fig. 10. The height is set at $\frac{3}{4}$ in. below the top of the overflow tube to give maximum conditions for foam breaking. Air, which enters two holes in the hollow shaft in the centre of the gland (Fig. 11), is dispersed through the holes in the body of the eight blade impellor (Fig. 12). The top of the impellor blade is situated

$\frac{1}{8}$ in. below the cylindrical body of the down-draught tube, giving maximum circulation and aeration at this point" (Munro, 1964). The impellor shaft was driven by an A.C. wound Paravalux electric motor of 0.1 H.P., capable of 1,400 r.p.m., but when the fermentor contained medium, the speed was reduced to 1,200 r.p.m. Being A.C. wound the speed could not be reduced without a subsequent torque decrease.

Inoculation Procedure

A 2-day culture of hydrocarbon adapted yeast was used as inoculum. The slow growing Rhodotorula species were not used. Twenty ml. of culture was injected aseptically through the rubber septum on the vessel lid. Five hundred ml. of salts solution and 10 ml. hydrocarbon were added and the culture grown under batch conditions for 1 day with an air flow of 600 ml./min. Fresh medium was then added continuously at a dilution rate of 0.1 $hr.^{-1}$.

Separation of Free and Attached Cells

Cells attached to hydrocarbon droplets were separated by repeated fractionation and washing. Five hundred ml. of batch culture were poured into a 500 ml. graduated flask and allowed to stand. When the hydrocarbon/yeast emulsion had filled the flask neck, this upper phase was carefully removed by pipette and resuspended in 500 ml. of fresh medium. This process was repeated until microscopic examination showed 20-30 free cells

present per field.

Protein Estimation

The protein content of 4 isolates (2 pigmented and 2 non-pigmented), growing in batch culture with varying percentages of hydrocarbon, was determined by the micro-Kjeldahl method of nitrogen estimation. The hydrocarbon in the media used was 1.2, 0.8 and 0.4% v/v.

After 3 days growth, cells were separated from residual hydrocarbon by Millipore filtration and dried at 104° for 2 days. Cells, still attached to the membrane, were weighed and digested in pure H_2SO_4. The NH_3, produced in the subsequent NaOH distillation stage, was trapped in standardised HCl solution and measured by titration with standard NaOH solution, with phenolphthalein indicator. Unused membranes were similarly treated and an allowance made in calculations for the nitrogen content of the membrane. All estimations were performed in duplicate and a mean taken. The accuracy of the technique was checked by using bovine serum albumen as a pure protein standard. Crude protein content was calculated by multiplying the nitrogen percentage by 6.2.

Table 4

Effect of pH on Growth

Isolate* group	pH values 3.0	3.5	4.0	4.5	5.0	5.5	6.0
A						+	
						++	+
B					+++		
					+++	++	+
C					+	+	++
					+	+	++
D (1)				+	+	++	++
				+	+	++	++
D (2)				+	+	++	++
				+	+	++	++
Pig (1)	+	+	+++	++++	+++	+++	
	+++	++++	++++	++++	++++	++++	
Pig (2)		+	+	+	+	+++	
		+++	+++	+++	+++	+++	
Pig (3)		+	+	++	+++	++	
	+	++	++++	++++	++++	++++	

* SEE p 48.

+ ~~slight growth~~
++ ~~growth~~
+++ ~~good growth~~
++++ ~~excellent growth~~

} RELATIVE AMOUNTS OF GROWTH

The upper value is the reading after 2 days and the lower is the reading after 4 days growth.

Table 5

Effect of pH on Growth Rate measured as a Change in pH

Isolate group *	pH values 3.0	3.5	4.0	4.5	5.0	5.5	6.0
A	0.6	0.3	-0.15	-0.35	-0.6	-0.85	-0.8
B	0.3	0.1	-0.2	-0.7	-1.85	-1.35	-1.0
C	0.2	0.7	-0.3	-0.3	-0.7	-1.1	-1.0
D (1)	0.4	0.5	0.45	0.6	-0.2	-0.65	-0.5
D (2)	0.4	0.1	-0.1	-0.45	-0.7	-1.4	-0.9
Pig (1)	-0.2	-0.4	-1.1	-1.5	-1.9	-2.5	-3.0
Pig (2)	0.4	0.1	-0.1	-0.45	-0.7	-1.4	-0.9
Pig (3)	0.15	-0.3	-0.9	-1.3	-1.5	-2.4	-2.8

* SEE p 48.

Table 6

Effect of Trace Element Solution Concentration upon Growth Rate

Isolate group *	Trace Element Solution Concentration (ml./l.) 0.05	0.025	0
B	++	++	+
	+++	+++	+
D (1)	+	+	+
	+	++	++
D (2)	+	++	+
	+	++	++
D (3)	+	+	+
	+++	+++	+
D (4)	+	+	+
	++	++	++
Pig (1)	-	+++	-
	++	+++	++
Pig (2)	-	+++	+++
	+	+++	+++
Pig (3)	-	+++	++
	+	+++	+++

The upper result refers to growth on day 2, the lower to growth on day 4.

* SEE P 48.

RESULTS AND DISCUSSION

Medium modifications

Growth on the original hydrocarbon salts medium was poor and an attempt was made to improve growth rates and yields.

pH variation

The pH of the medium was varied from 3.0 to 6.0 and the effect on growth rate observed (Table 4). The results of readings taken after 2 days showed that the optimum pH value for growth rate lay between 5.0 and 6.0. A pH value of 5.5 was used in all future experiments. This pH optimum was also indicated by the changes of pH occurring (Table 5), since good growth was accompanied by a large fall in pH. The rise in pH of certain media in which there was little growth was shown to be an effect of sterilisation. Regardless of the initial pH of the medium, there was a rise of 0.2 - 0.5 pH units after autoclaving. Since there was no single inorganic buffer which operated over the entire pH range, pH was adjusted by acid or alkali addition, and pH readings taken before inoculation.

Trace elements concentration

It would seem from Table 6 that these ions were not essential for growth, or, more probably, that the other constituents of the medium contained sufficient amounts of them. Though they were not essential, they were still added at a

Table 7

Effect on Growth of Yeast Extract Supplementation

Isolate Group*	No Yeast Extract	Yeast Extract
A	+	++++
B	+	++++
C	+	++++
D (1)	++	++++
D (2)	++	++++
Pig (1)	++	++++
Pig (2)	++	++++
Pig (3)	++	++++

* SEE p 48.

Table 8

Effect of Growth Factors upon Growth Rate

Concentration of Growth factor (mg./ml.)	1×10^{-2}	1×10^{-3}
All Growth Factors	-	-
Group (1) Growth Factors	-	-
Group (2) Factors	+++	++
Folic acid	+++	++
Riboflavin	+	+
Pyridoxine	+	+
Biotin	+	+
Thiamine	+	+
Nicotinic acid	+	+
Pantothenate	+	+
Inositol	+	+
Ascorbic acid	+	+
Cobalamine	-	-

0.01% Yeast Extract ++++

Unsupplemented medium +

Group (1) - Pantothenate, nicotinic acid, pyridoxine, thiamine and cobalamine

Group (2) - Folic acid, riboflavine, biotin, inositol and ascorbic acid

\+ slight growth

+++ very good growth

++++ excellent growth

concentration of 0.025 ml. per 1. salts solution, lest some element became growth limiting during profuse batch culture.

Yeast extract supplementation

Even after adjustment of H^+ ion and trace elements concentrations the growth in the basal salts hydrocarbon medium remained RELATIVELY poor. It was therefore supplemented with yeast extract. When 8 of the isolates were grown in this medium all showed greatly enhanced growth (Table 7).

Specific growth factor(s)

This experiment was an attempt to find the specific growth factor(s) in the yeast extract responsible for the improved growth (Table 8). Of all the growth factors, only folic acid stimulated growth: Cobalamine, at the concentrations employed here, was inhibitory.

The organism must possess some capacity for synthesising folic acid, otherwise growth in the hydrocarbon salts medium would not occur. Supplementation with this growth factor allowed much faster growth than was previously possible. Comparison of growth rates in the presence of folic acid and yeast extract, showed that the latter was more effective; subsequently the medium was supplemented with yeast extract.

Table 9

Classification Results

Classification Group	A	B	C	D	Pigmented
Number of Isolates	1	3	8	13	3
Pseudomycelium Production	-	-	-	-	+
Pellicle Formation	-	-	-	-	+
Pigmentation	-	-	-	-	+
Sugar Fermentation					
Glucose	+	+	+	+	-
Galactose	+	-	-	-	-
Sucrose	+	-	+	+	-
Maltose	+	+	+	+	-
Lactose	-	-	-	-	-
Raffinose	+	-	-	+ or -	-
Sugar Utilization					
Glucose	+	+	+	+	+
Galactose	+	+	-	-	-
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	-	+	-
Nitrate Utilization	-	-	-	-	+
Ethanol Utilization	-	-	-	-	-
Arbutin Splitting	-	-	-	-	-

Classification

The method of taxonomy used was that of Lodder and Kreger-van Rij (1952). Yeasts were assigned to a particular family on the basis of the presence and morphology of their sexual phase. Further classification to the levels of genera and species depend on morphological and biochemical characteristics.

Morphology

All 25 non-pigmented isolates had a similar morphology with only slight variations in cellular dimensions. The cells tended to be short oval, to oval in shape, single or with one terminal or slightly sub terminal bud. The dimensions were 4 - 6 μ by 2 - 3 μ. The morphology of the 3 pigmented isolates was similar. The cells were 6 - 12 μ by 2 - 3 μ. Usually they grew singly or in pairs. Occasionally short chains of 3 - 4 cells were formed.

The colonial morphology of the non-pigmented isolates was similar, the colonies being whitish, domed, shiny and soft in consistency. The diameter was 3 - 4 mm. The colonies of the pigmented isolates were similar to each other, having a very rough, dry appearance with horn-like projections. They were extremely resistant to fragmentation. Colony diameter was 1 - 2 mm.

The results of the other classification tests are shown in Table 9. Because of the absence of any sexual phase, all isolates were assigned to the Cryptococcaceae. The pigmented

strains were assigned to the genus Rhodotorula and all the non-pigmented isolates to the genus Torulopsis.

The Torulopsis isolates (groups A, C and D) differed only slightly in their biochemical properties. Only A fermented galactose, and only C was unable to utilize lactose. As it was impossible to assign any of these groups to any known species, they were regarded as being strains of a new species intermediate in its properties among the T. colliculosa, holmii, dattila, gropengiesseri and versitalis complex. The isolates of group B were sufficiently similar to T. etchellsii to merit classification as strains of this species, although they utilized sucrose and lactose, and failed to assimilate nitrate, unlike the type species. However, only 2 strains were examined by Lodder and Kreger-van Rij (1952), so these variations may not be important.

Despite the inability of the Rhodotorula isolates to assimilate galactose, their morphology and other biochemical tests suggested that they were strains of a new species occupying an intermediate position between R. aurantiaca and R. rubra.

The results of this study are partly supported by the work of Scheda and Bos (1966) who found Rhodotorula and Torulopsis species, particularly T. dattila, to be n-paraffin utilizing organisms. Takeda, Iguchi, Kawamura and Horiguchi (1965) isolated 7 hydrocarbon assimilating yeasts, 4 Candida, 1 Torulopsis and 2 Brettanomyces, all of which were new species.

General Discussion

No species of the genus Candida were isolated. It seems unlikely that no mud and water samples held Candida yeasts, yet during enrichments only Torulopsis species were found. It is known that Candida requires yeast extract supplementation when grown on salts/hydrocarbon medium, so it would seem likely that the absence of growth factors in the enrichment medium prevented isolation of Candida species. It is unlikely that the pH value was inhibitory, since the optimum pH for Candida growing on hydrocarbons is 5.5 (B.P., private communication). It is also improbable that the Torulopsis merely outgrew the Candida, since a Rhodotorula isolate, a slow growing yeast, was obtained from the same enrichment as a Torulopsis isolate.

Substrate Availability Studies

As the n-paraffin fraction used was virtually insoluble in water (Fig. 9), the means by which the yeast cell obtained the hydrocarbon was studied. Two possibilities exist, either the cell grows on minute traces of hydrocarbon dissolved in the aqueous phase, or physical attachment to the substrate is necessary. If the former mechanism operates, it could be envisaged as occurring by one, or both of the following methods. Either the cells were growing on dissolved hydrocarbon, causing a permanent imbalance in the equilibrium between the two phases; or an extracellular enzyme converts the n-paraffins to a more water-soluble substrate, which the cell then metabolises.

If, however, contact between cell and substrate were necessary, either one, or both, of the following mechanisms could operate. An enzyme bound to the cell wall may modify the substrate and enable it to pass through the wall; or perhaps the hydrocarbon may become dissolved in some component of the wall and diffuse inwards to the cytoplasmic membrane.

The following experiments were designed to examine the mode of substrate utilization.

Growth on Dissolved Hydrocarbon

This test was performed to examine the possibility of growth being at the expense of dissolved hydrocarbon. A solution of the hydrocarbon was prepared by shaking the salts solution and hydrocarbon together and separating the aqueous

phase. This solution, and untreated salts solution, acting as a control, were inoculated and growth observed. Slight growth occurred in the test vessel and none in the control. This result suggests that physical contact was necessary. The slight growth could have been at the expense of minute hydrocarbon droplets which had not been separated from the aqueous phase; or, alternatively, traces of dissolved substrate, insufficient for prolonged growth, could have been present.

If the last suggestion were true, then profuse growth would occur in a system where the hydrocarbon was out of contact with the cells, but where it was still in contact with the aqueous phase to supply fresh dissolved substrate constantly. The second experiment was an attempt to set up such a system. Here the hydrocarbon was contained within dialysis tubing which floated in the salts solution. The aqueous phase was inoculated and growth observed. After 2 days there was profuse growth in the aqueous phase, but microscopic examination showed hydrocarbon droplets also present. The dialysis tubing had been too permeable to the hydrocarbon, which had formed droplets outside the membrane. Ideally, a barrier was required which would very slowly release the substrate at a rate equal to, or less than, that necessary for growth. As it seemed unlikely that such a membrane existed, experiments involving this approach were discontinued.

To test further the idea that growth occurred at the expense of dissolved hydrocarbon, a third experiment was set up.

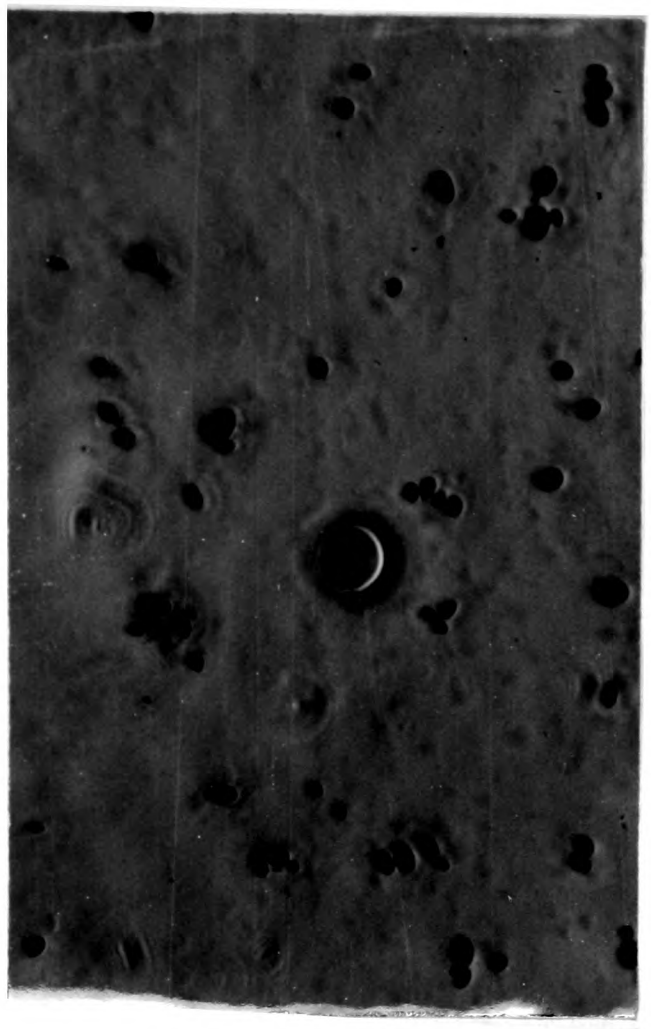


Figure 13

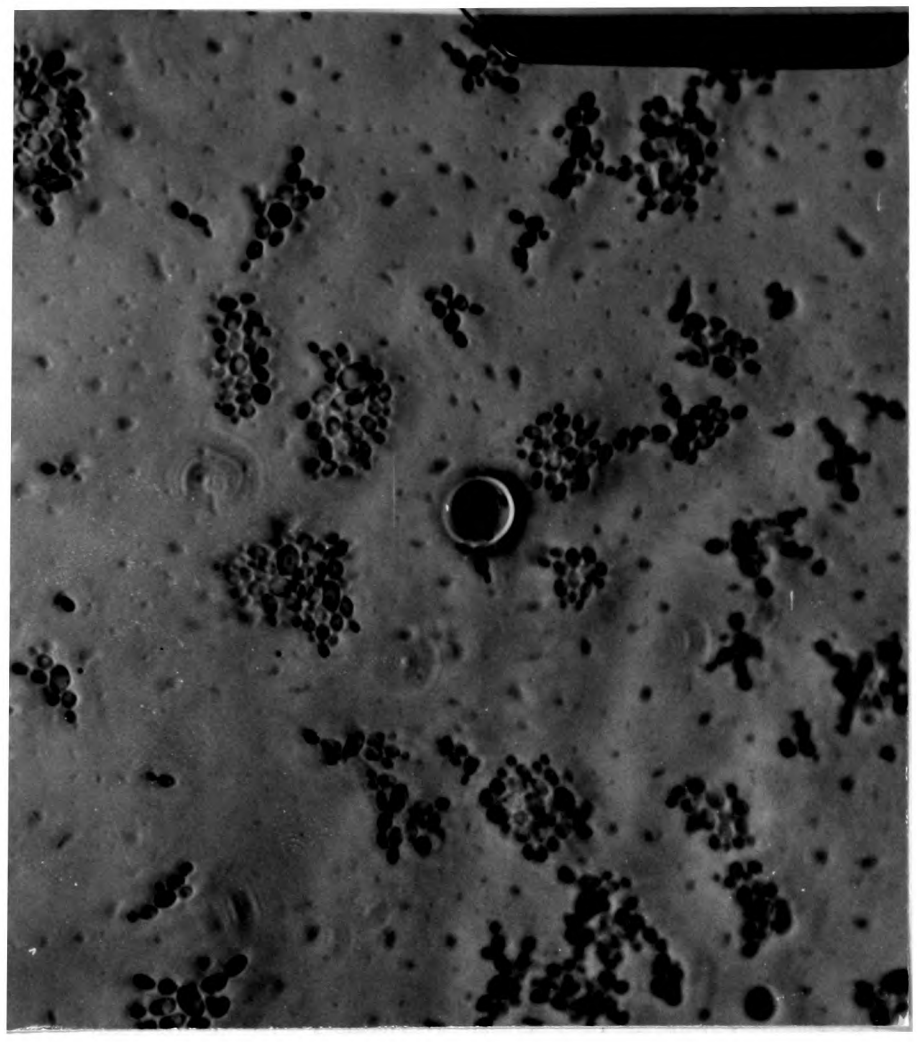


Figure 14

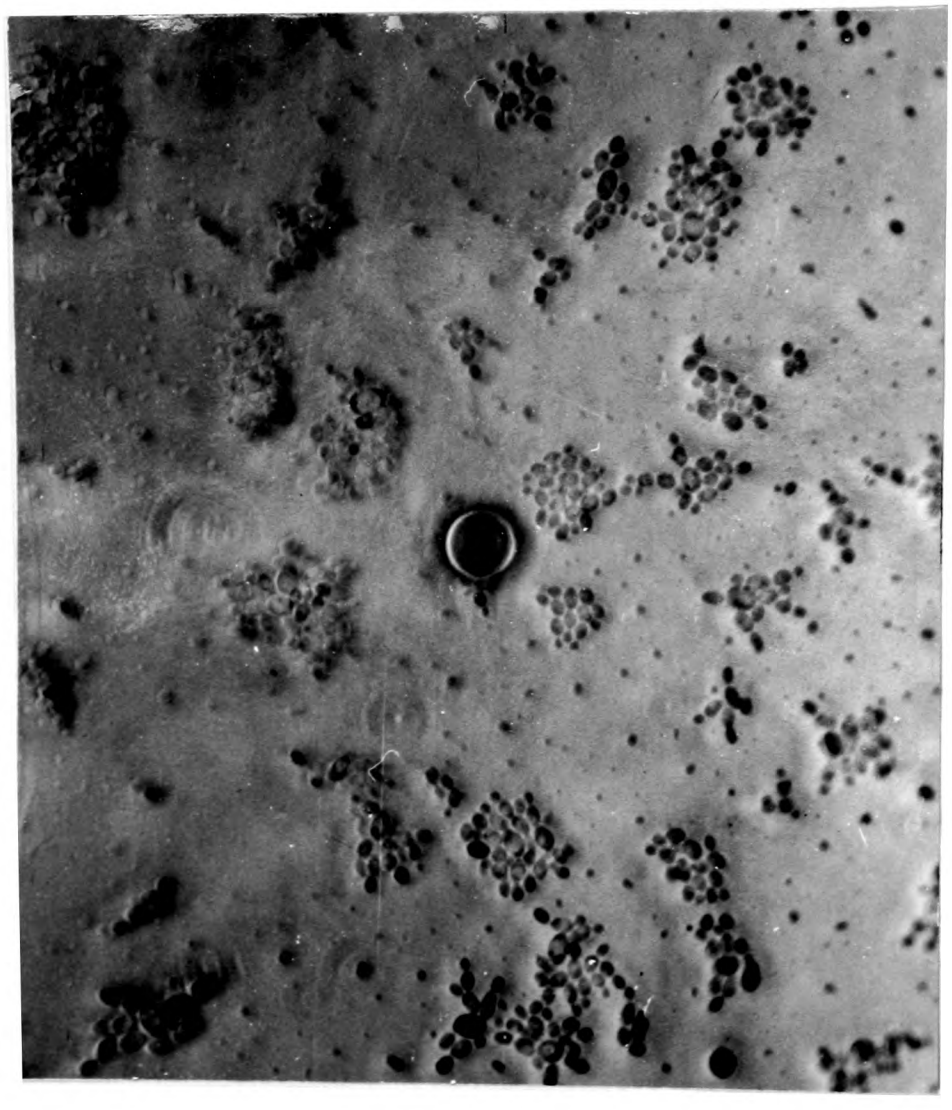


Figure 15

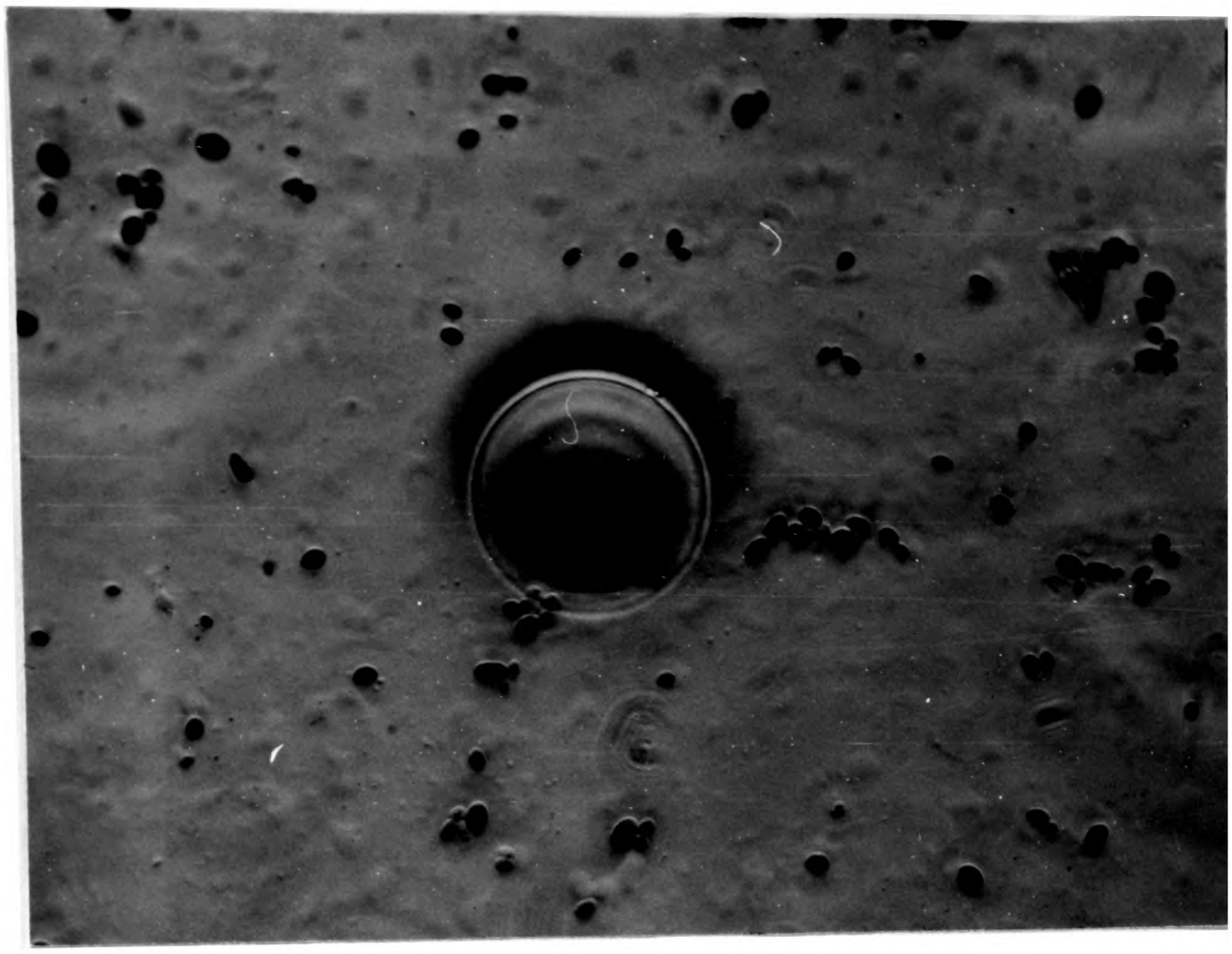
Failure of cells to grow although in close proximity to hydrocarbon droplet

Fig. 13 1 hr.

Fig. 14 20 hr.

Fig. 15 241 hr.

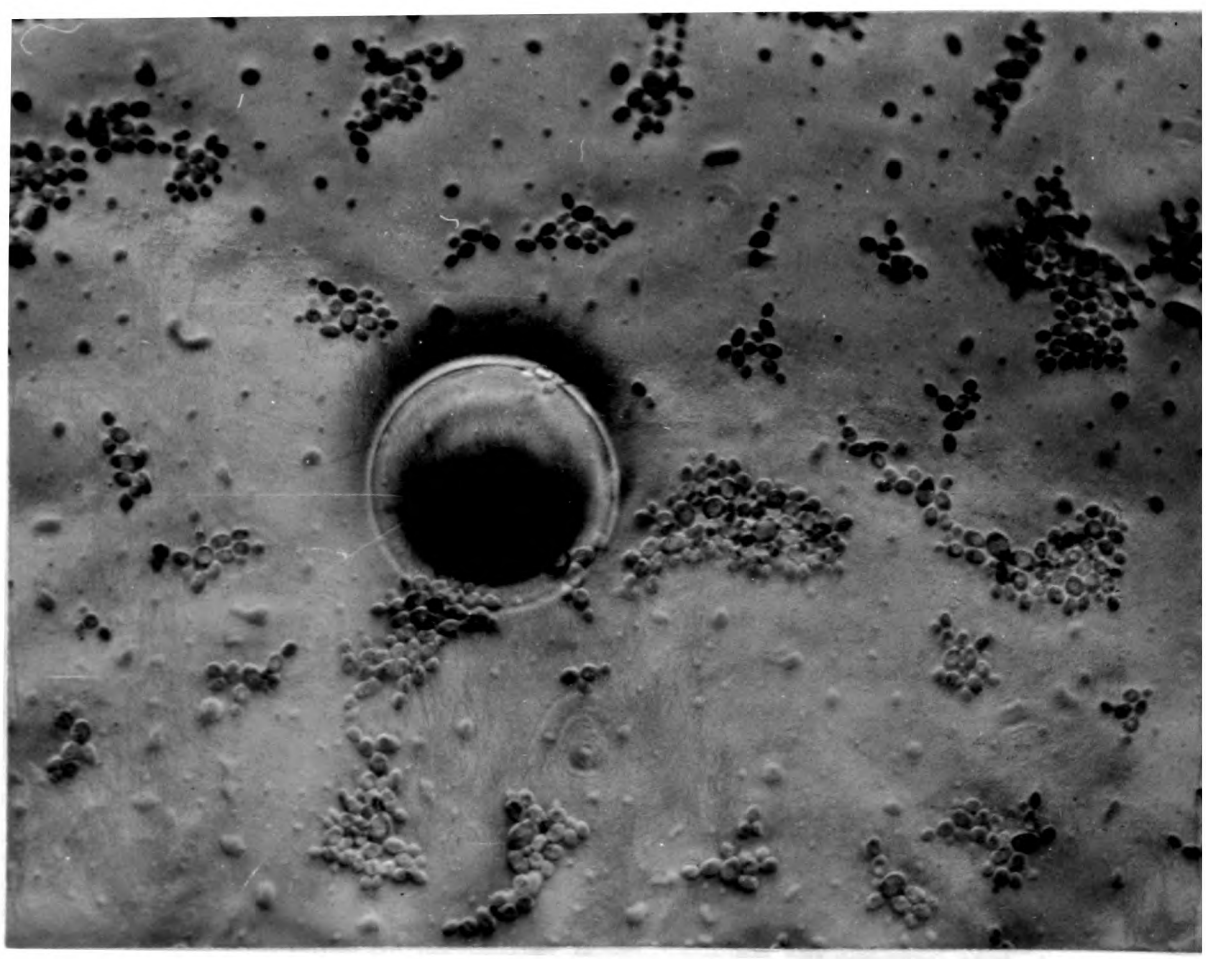
Magnification 470 x



1 hr.

Figure 16

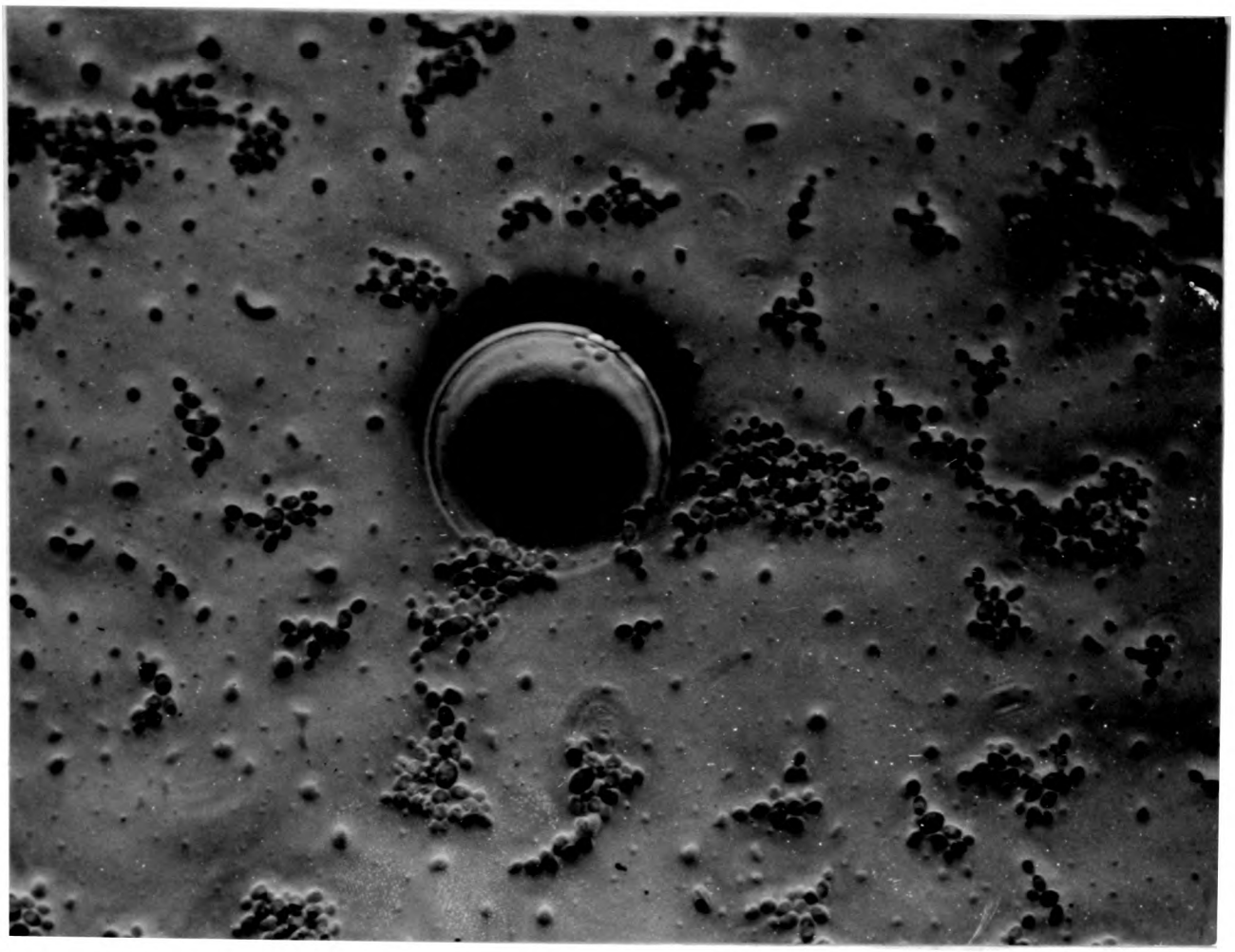
Figs. 16 - 21 show growth sequence of cells attached and unattached to hydrocarbon droplet



21 hr.

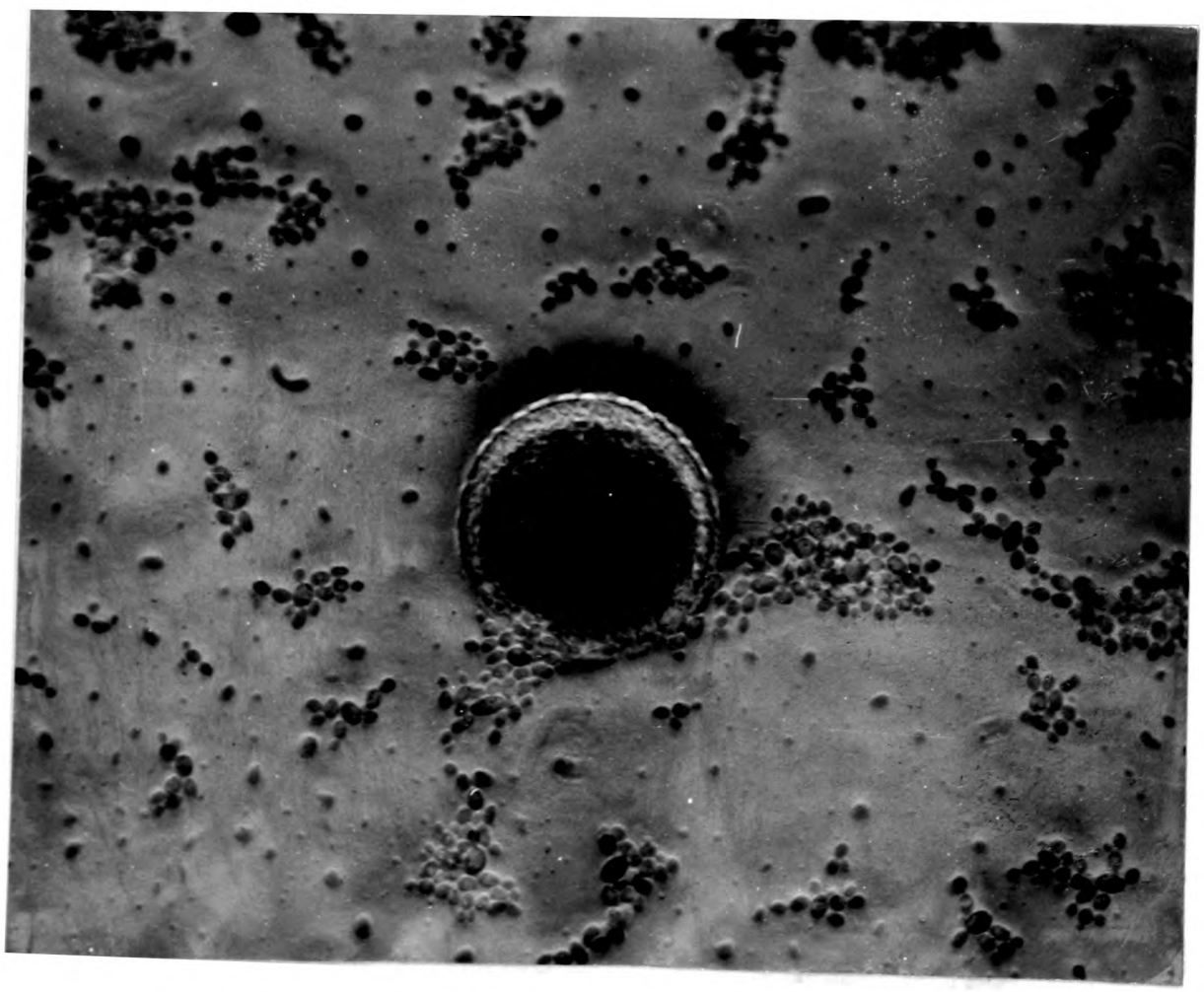
Figure 17

Magnification 470 x



45 hr.

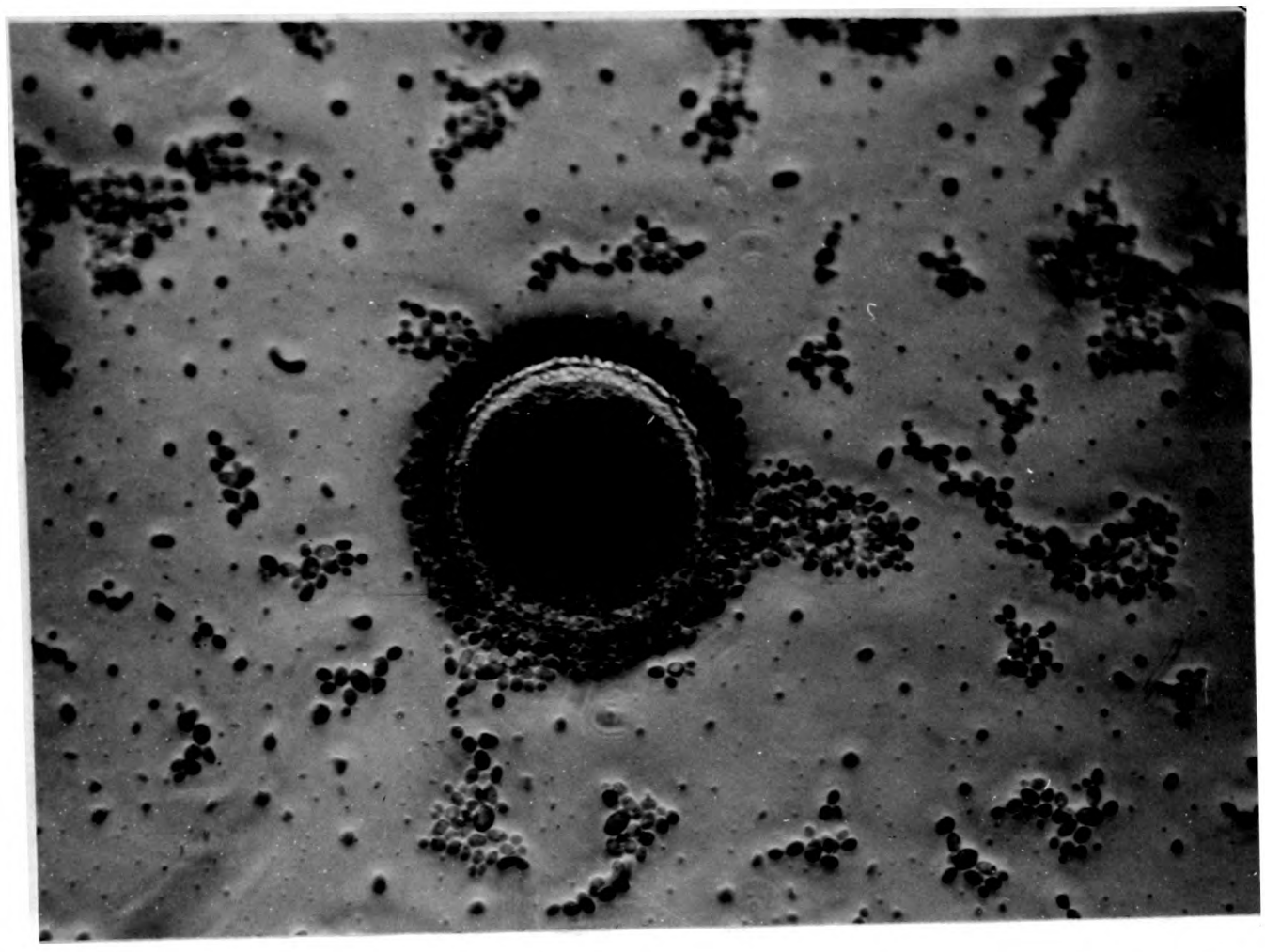
Figure 18



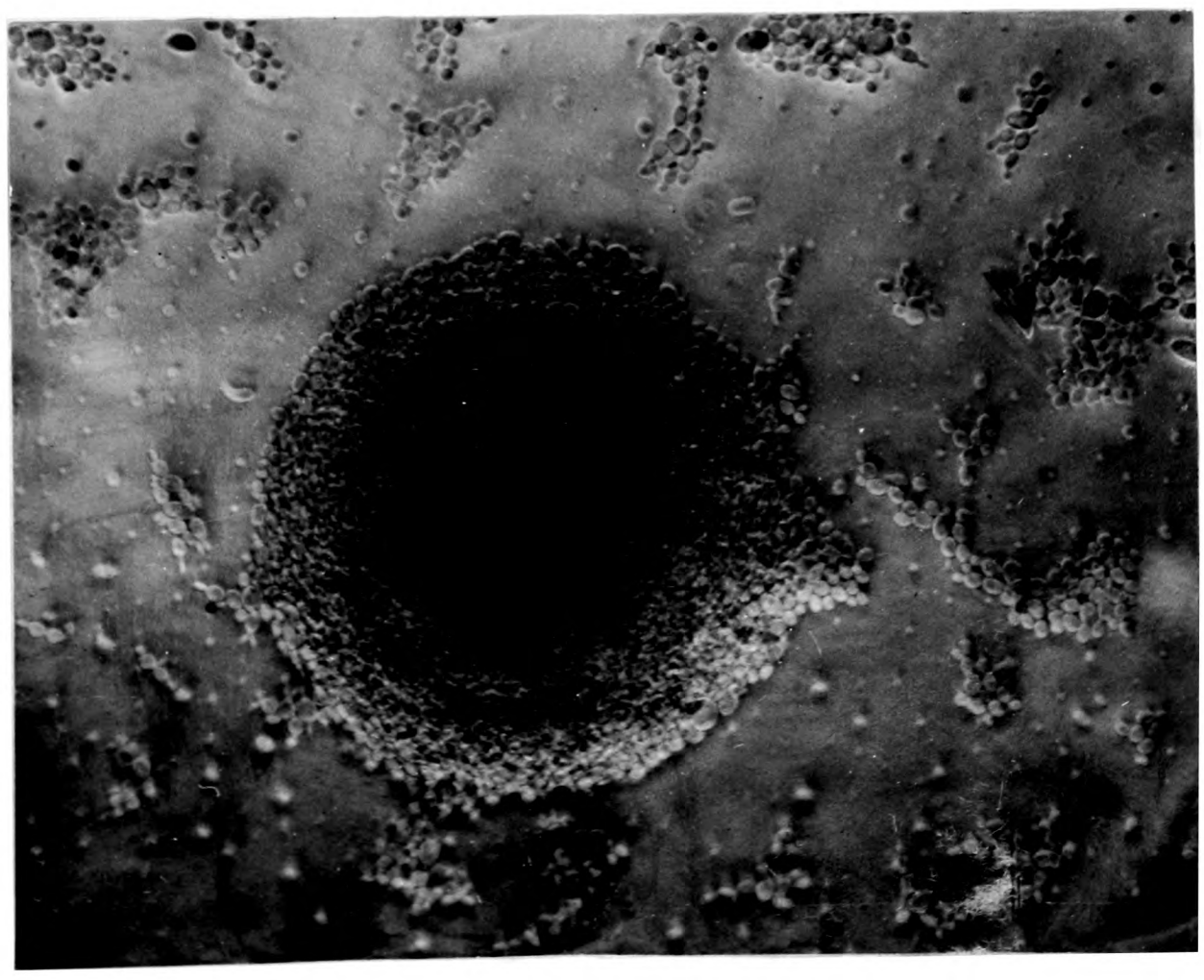
69 hr.

Figure 19

Magnification 470 x



93 hr.



169 hr.

Figure 21 Magnification 470 x

The aqueous phases of medium in 2 vessels were saturated with dissolved hydrocarbon by adding volumes of hydrocarbon differing by a factor of 10. After inoculation growth was observed. If growth occurred on dissolved substrate, then equal amounts of growth would be expected. This did not happen. The vessel containing the greater amount of hydrocarbon contained more growth.

This experiment strongly suggested that growth required physical contact with the substrate. The following series of experiments were performed to verify this suggestion.

Slide Culture

Using slide culture techniques, growth on hydrocarbon droplets was observed over several days. Only those cells actually touching the droplet proliferated continuously, whilst the others budded 3-4 times then ceased (Figs. 13-21). The behaviour of cells in close proximity to a droplet upon which there was profuse growth, indicated the possible mode of entry of substrate into the cell. If the n-paraffins were converted to a water-soluble compound by some extracellular enzyme(s), better growth of cells closer to the droplet would be expected, since some diffused nutrient would be available. However, this was not observed. All unattached cells, regardless of their proximity to the droplet, budded only 3-4 times, presumably at the expense of traces of nutrient in the agar and reserve materials. SIMILAR BEHAVIOUR WAS NOTED IN HYDROCARBON-FREE PREPARATIONS. Thus it is reasonable to postulate that

Table 10

Optical Density of Hydrocarbon/Pristane Emulsions

Percentage n-paraffin (v/v)	Percentage pristane (v/v)	Mixture	Extinction
83	17	A	0.26
17	83	B	0.56
100	0	C	0.20
0	100	D	0.68

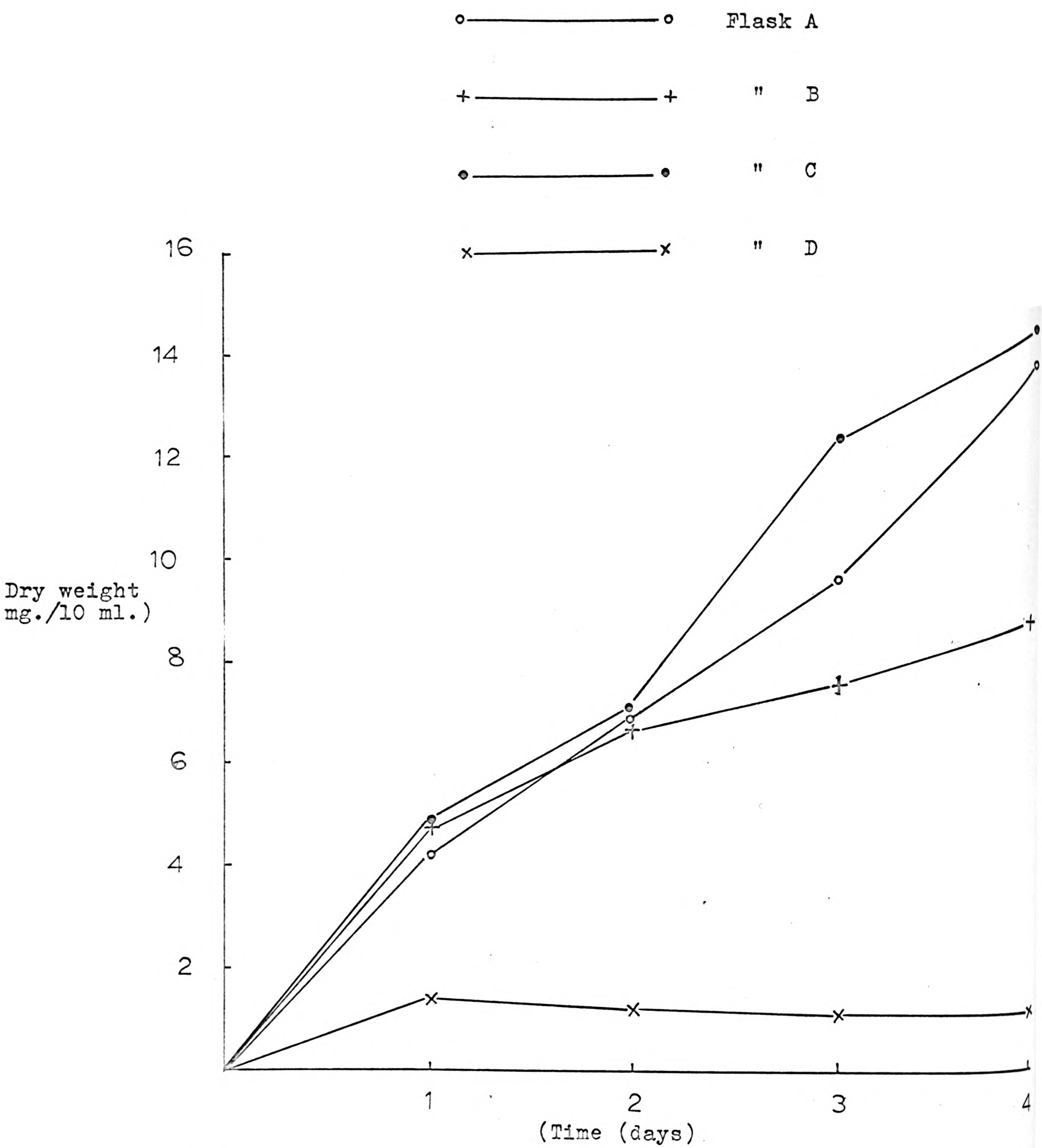


Figure 22

Growth rates of yeast growing on different n-paraffin concentration

n-paraffins enter the cell unaltered.

Effect of Substrate Availability on Growth Rate

Since physical contact is essential for growth, it is also likely that the rate of growth depends rather upon the surface area of the substrate than on the actual amount of substrate present. A fifth experiment was set up in which the amount of hydrocarbon was varied, but the volume, and subsequently the surface area remained constant. This was effected by diluting the n-paraffins with a biologically inert hydrocarbon, pristane, the branched nature of its carbon skeleton rendering it unsuitable for metabolism. An important assumption in this experiment was that incubation emulsified the different n-paraffin dilutions to a similar degree. The tendency of the different mixtures to emulsify was measured by blending samples in medium in an osteriser jar and measuring the optical densities of the emulsions (Table 10). Solutions, containing a large proportion of pristane, had a greater tendency to emulsify during blending than those with little or no pristane present. However, it was thought unlikely that gentle agitation experienced during incubation would effect the dispersion of the hydrocarbon mixtures to such a degree.

Four growth vessels (A to D) were set up and equal volumes of the different hydrocarbon dilutions were added to each. The growth rates were measured by determining the dry weight of samples taken daily (Fig. 22). Growth in all vessels

containing n-paraffin was the same after 2 days, after which time rapid growth continued only in those flasks initially containing high percentages of n-paraffins (flasks A and C). In flask B lack of substrate began to limit growth after 2 days. Flask D served as a growth control for pristane. Growth rates in flasks A to C were linear.

The observation, that growth was similar after 2 days in flasks A to C, supported the belief that the different emulsification tendencies of the hydrocarbon mixtures were unimportant during incubation. This observation also proved that, provided n-paraffin was not limiting, growth rate was independent of the amount of substrate, and governed by its surface availability only.

An interesting feature of these results was the linear growth, particularly in flasks A and C. Since ample substrate remained, the linearity of growth rate must be attributed to limiting availability of substrate. Slide culture studies have shown the necessity for attachment to the droplet if growth was to continue. It would seem that, in liquid culture, only those cells attached to the droplets were proliferating and shedding their progeny into the medium at a constant linear rate. This situation would continue until the droplets become reduced in volume and surface area, and the growth rate would decrease accordingly.

As a corollary of the importance of physical contact between cell and substrate, an explanation of the enhancing

effect of yeast extract on growth rate can be suggested. As well as supplying folic acid, the soluble nutrients would encourage initial growth of the inoculum before contact with the hydrocarbon was established. Also, the yeast extract assisted in the preliminary emulsification of the hydrocarbon during early incubation.

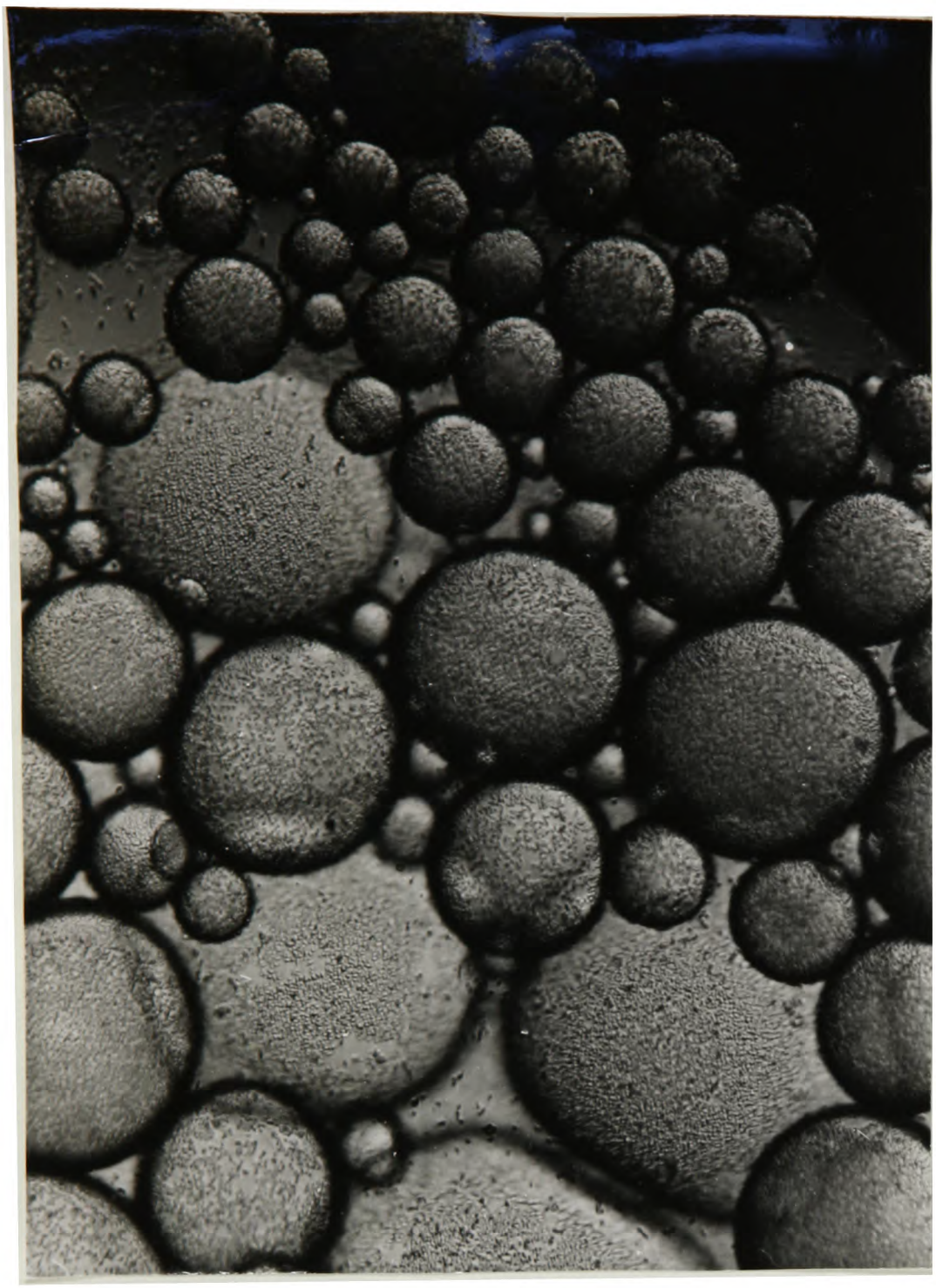


Figure 23

Suspension of batch-grown cells attached to hydrocarbon droplets

Magnification 150 x

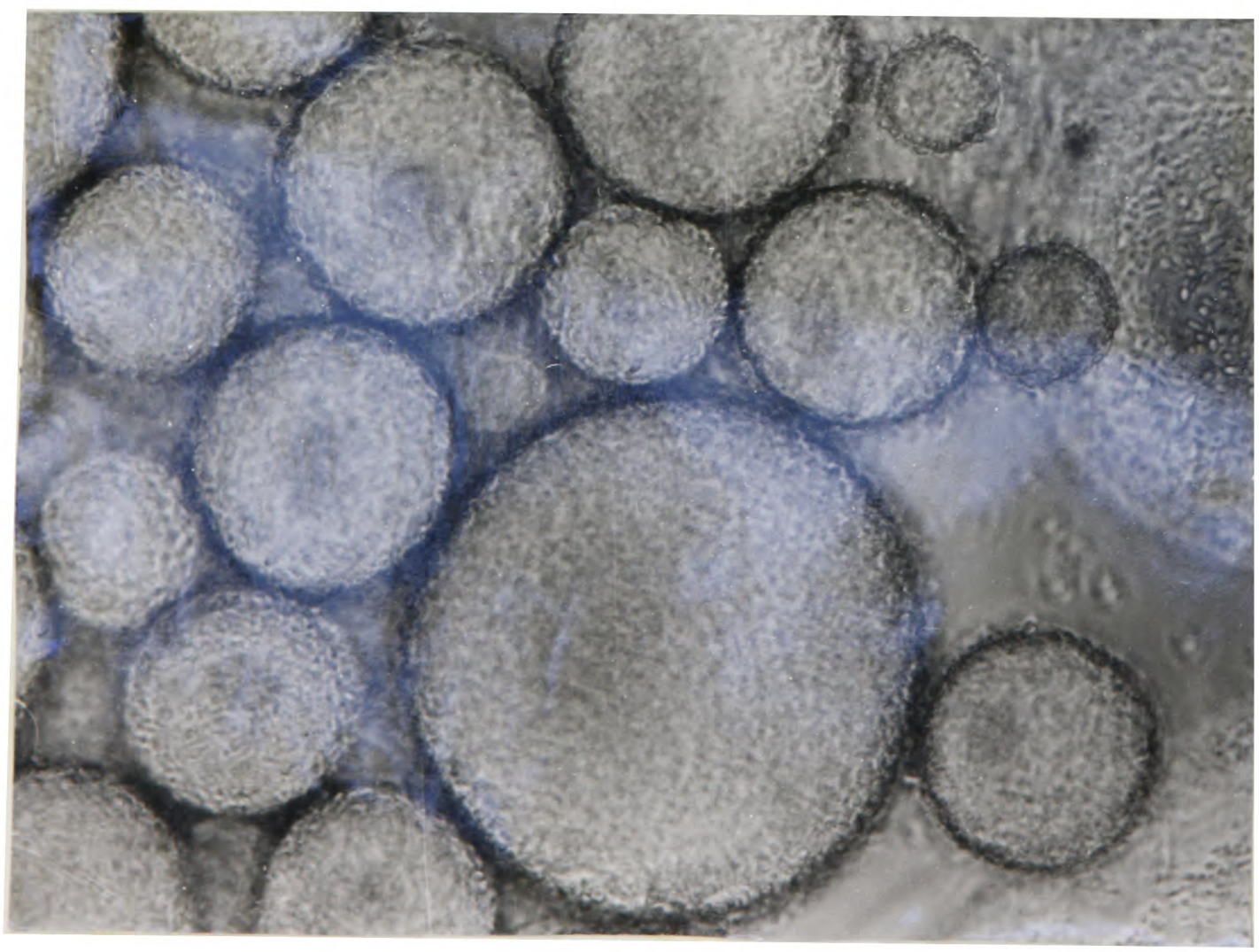


Figure 24

As above

Magnification
470 x

Figure 25

Separation of cells from hydrocarbon droplets after agitation in the chemostat for 10 sec.

Magnification 470 x

Continuous Culture Studies

An attempt was made to grow yeast on hydrocarbon in continuous culture to evaluate growth rates and yield factors in the constant environment of the chemostat.

Microscopic examination of samples withdrawn during continuous culture showed numerous small hydrocarbon droplets and a few unattached cells. Since the growth was so poor, it was thought that some component of the vessel's structure had a toxic effect by dissolving in the medium. However, samples aseptically withdrawn grew profusely in overnight batch culture in shake-flasks. Another possible reason for poor growth may have been the high oxygen tension, resulting from the rapid air flow rate and high stirring speed. A reduction of the air flow to 100 ml./min. did not improve growth in the vessel.

Since availability studies had shown the importance of physical contact between cell and substrate, the conditions in the chemostat may be such as to prevent attachment to the droplets. This theory was tested as follows. Yeast cells attached to hydrocarbon droplets were separated from free cells in batch culture by repeated fractionation and washing. The emulsion was carefully added to the fermentor and the impellor switched on. Samples, withdrawn after 10, 30, 60 seconds and 5 minutes, were examined microscopically. After 10 seconds the droplet diameter had been greatly reduced and few cells remained attached (Figs. 23-25). The sample taken after 5 minutes was no different from the others, suggesting that

equilibrium between attached and free cells was rapidly achieved. It was concluded that the failure of the yeast to grow in continuous culture was directly attributed to lack of contact with the hydrocarbon. This then caused a reduction in generation time, allowing "wash-out" to occur even at low dilution rates. Removal of the down-draught tube improved growth conditions by allowing better contact between cell and substrate, but growth remained poor. Since the design of the fermentor assembly was such as to render it inflexible to further modifications which would remove the excessive shearing, further continuous culture studies were discontinued.

Table 11

Crude Protein Content of Cells Grown in Media of Varying Hydrocarbon Content

Isolate	Percentage (v/v) Hydrocarbon in Medium		
	1.2	0.8	0.4
D (1)	79	87	60
D (2)	71	60	69
Pig (1)	58	56	50
Pig (2)	21	30	31

(% dry weight)

Table 12

Mean Protein Content of Yeasts Grown in Media of Varying Hydrocarbon Content

Isolate	Mean Protein Content (% dry weight)
D (1)	75
D (2)	66
Pig (1)	55
Pig (2)	27
B.P. Candida	62-66
Carbohydrate grown Torula yeast	51

Protein Content

Since no values for the crude protein content of hydrocarbon-grown Torulopsis and Rhodotorula species appeared in the literature, the protein content of 4 isolates grown in media with differing hydrocarbon content, was estimated (Table 11). The results were compared with those obtained by British Petroleum with a Candida species (Table 12). Here the mean is shown of the 3 protein percentage figures obtained from cells grown in the different media.

No relationship was found between protein content and the amount of hydrocarbon in the medium. There was a noticeable difference between the protein contents of the Torulopsis and Rhodotorula strains. This was probably due to the tendency of Rhodotorula species to store lipid. Comparison of the protein content of Torulopsis strain D(1), with that of commercially grown Candida showed 9% more protein. However, a control experiment, using a pure protein, showed a tendency for the technique to have an error of plus 1 - 5%. Even so, with a maximum error of 5%, this isolate would still contain 8% more protein. A fairer comparison between the two genera would be to grow D(1) in continuous culture and estimate the protein content, since the mean figure of 75% is obtained only from 3 results, one of which is very high (87%). This may be caused by the conditions of the batch culture being such as to reduce the lipid content of the cell, thus enhancing the protein dry weight.

Applied Aspects of Substrate Availability

The prerequisite for physical contact and the correlation between surface area and growth rate have been tacitly assumed by many workers, but little direct evidence has been presented. Ladd (1956) noted similar oxygen uptake rates with cells growing on different concentrations of n-decane in the medium, the amounts of substrate differing by a factor of five. In the system containing the lesser volume of hydrocarbon, the reduction in substrate area was compensated for by supplying the hydrocarbon in an emulsion.

The findings of this report have shown the strict necessity for physical contact between cell and substrate if growth was to occur. The cells' failure to grow under high shearing conditions in the chemostat might suggest that contact must occur for a finite time. If this period were too brief, then insufficient nutrient would enter the cell to sustain growth, and wash out would occur.

The previously described heterogeneity of a population growing on n-paraffins suggests several interesting features in the fields of biomass production and fermentor design, since some of the cells exist in a state of carbon deficiency when unattached to droplets. Homogeneity would only be achieved when the interchange between attached and free cells proceeded at a rate permitting time for the entry of sufficient nutrient to sustain exponential growth. For such a situation to occur, it would be essential to have a large substrate area available

at all times. This could be achieved in two ways. Hydrocarbon and aqueous medium, before entering the vessel, could be pre-emulsified to such a degree that droplet size approached cellular dimensions, preventing coalescence when the emulsion was added to the main vessel. (Microscopic observations of a mixture of yeast cells and "micro" droplets have shown that a matrix of cells and droplets is formed in which the hydrocarbon is effectively prevented from coalescing.) A second method of providing available substrate would be to have a localised site in which a shearing effect stripped cells off droplets and gave free cells an opportunity of obtaining nutrient. As a result of these two features the expense of bulk mixing in the fermentor would be reduced, since the impellor system need only operate at a speed sufficient to disperse air throughout the culture.

Materials and Methods

Growth Temperature

Unless otherwise stated, incubation was at 30°.

Storage of Materials

Media and samples of mud for isolation studies were stored at 4°.

Sterilisation

Unless otherwise stated, all materials were autoclaved at 121° for 15 minutes.

Gaseous Hydrocarbons

The gases used were methane, ethane, propane and n-butane. With the exception of methane, which was obtained from Mogden Sewage L.C.C., the remaining gases were supplied by Air Products Ltd. Ethane was used in two grades of purity: technical grade 95% v/v pure and chemically pure (C.P.) grade, 99% v/v pure. Propane and butane were both C.P. grade. The impurities in any of these 3 gases were traces of the other two. Butane also contained a minute amount of iso-butane. Methane contained no other hydrocarbons as impurities. Gases were analysed using gas-liquid chromatography with flame ionisation detection.

Growth Techniques

Growth on solid medium in an atmosphere of hydrocarbon and air was effected by evacuating a large glass vacuum desiccator (6-7 l.) and adding 3-4 l. of hydrocarbon from a bladder. Air was then allowed to enter the vessel, but a slight negative pressure was left to ensure an effective seal. Occasionally Tupperware vessels were used, but with these containers there was no evacuation. Instead some of the air was displaced by blowing in a bladderful of gas.

Growth in liquid batch culture was achieved by using sealed 250 ml. conical Quickfit flasks containing 50 ml. medium. After inoculation, the sterile cotton-wool plug was removed and replaced with a sterile Suba seal. A small piece of cotton-wool in the centre core of the seal acted as a filter when the gaseous hydrocarbon was added. The gas (usually 50 ml.) was injected by syringe through the seal. Cultures were incubated on a Gallenkamp Orbital Shaker operating at 200-250 r.p.m.

Enrichment Media

Two types of media, differing only in their nitrogen sources, were used. The basal salts solution was prepared as follows: KNO_3, 1.0 g. or NH_4Cl, 0.5 g.; $MgSO_4.7H_2O$, 1.0 g.; $CaCl_2.6H_2O$, 0.1 g.; Sequestrine-iron complex C.P. 2 Geigy (Johnsons of Hendon Ltd.), 1.0 ml. of a 0.4% (w/v) solution; trace elements solution, 0.5 ml. were dissolved in 1 l.

distilled water. The pH was adjusted to 6.7 - 7.0 with 1 N - H_2SO_4 or 1 N - NaOH solution.

The trace elements solution contained $AlCl_3$, 1.0 g.; KI, 0.5 g.; KBr, 0.5 g.; LiCl, 0.5 g.; $MnCl_2.4H_2O$, 7.0 g.; H_3BO_3, 11.0 g.; $ZnCl_2$, 1.0 g.; $CoCl_2$, 1.0 g.; $NiCl_2$, 1.0 g.; $CoCl_2$, 1.0 g.; $SnCl_2.2H_2O$, 0.5 g.; $BaCl_2$, 0.5 g.; Na_2MoO_4, 0.5 g.; $NaVO_3$, 0.1 g.; Se-salt, 0.5 g. Each salt was dissolved separately in distilled water and the pH value adjusted to 6.8 - 6.9 before mixing. The final volume of the complete mixture was 18 l. and the pH value 3.0 - 4.0.

After sterilisation, when the medium had cooled, 20 ml. of phosphate buffer were added per l. This buffer was prepared as follows: $Na_2HPO_4.12H_2O$, 10.74 g. and KH_2PO_4, 3.9 g. were dissolved in 100 ml. distilled water, giving a buffer of pH 6.8.

Solid medium was prepared by adding Davis No. 2 Ion agar at a concentration of 1.5% w/v.

Enrichment and Isolation Procedure

Mud and water samples from polluted environments were used as inocula. Each sample was grown in both types of enrichment medium under partial pressures of ethane, propane and butane. One ml. of sample was pipetted into a medical flat containing 30 ml. salts solution and sealed with a Suba seal. Thirty ml. of a gaseous hydrocarbon were injected and the bottle incubated vertically. Enrichments were examined over a period of 2-3

weeks. Those showing a pellicle were taken to contain hydrocarbon-utilizing micro-organisms. Such bottles were vigorously shaken to disperse the pellicle, and a series of 10-fold dilutions in medium were made. One drop of each dilution was placed on agar and allowed to dry in. Plates were incubated in an atmosphere of hydrocarbon and air for 4-5 days before examination. Those colonies appearing first, or having a dense nature were selected and purified. Each isolate was checked for its ability to utilize hydrocarbons by comparing growths of slope cultures grown in the presence and absence of hydrocarbon. Stock cultures were kept on agar slopes in a hydrocarbon/air atmosphere.

Colonial Morphology

The colonial morphology of isolates was observed using a plate microscope. Organisms were grown on nitrate medium using the hydrocarbon upon which they were isolated. Observations were made after 5-7 days and a second time after 12-14 days.

Cellular Morphology

Observations of morphology and motility of cultures growing on slopes were made using phase contrast microscopy. Dimensions were measured by an objectmicrometer. Acid-fast stains were made by the Ziehl-Neelsen method (Mackie and McCartney, 1962).

Biochemical and Physiological Properties

Growth at elevated temperature. The ability of the isolates to grow at temperatures of 33, 37, 40 and 45° was observed. Agar slopes in 6 x $\frac{5}{8}$ in. test-tubes were inoculated and the protruding pieces of the cotton-wool plugs were cut off. The remaining pieces were pushed 1 cm. down the neck and the tube closed with a Suba seal. Five ml. of hydrocarbon upon which the organisms were isolated, were injected and the cultures incubated for 6 days in water baths.

Urea utilization. The nitrogen source in the medium was replaced by 0.5% (w/v) urea. Isolates were streaked on to agar medium in Petri dishes, and incubated for 6 days under the gaseous hydrocarbon upon which they were isolated.

Nitrate utilization. All organisms originally isolated on ammonium medium were tested for the ability to grow on nitrate medium, with the gas upon which they were first isolated.

Gaseous substrate spectrum. The isolates were streaked on to nitrate agar in Petri dishes and grown in atmospheres of the different gaseous hydrocarbons in desiccators. Methane, and H_2 were also tested as possible substrates. Controls, incubated in air, were set up. All plates were examined after 7 days.

n-paraffin utilization. Isolates were streaked on to nitrate agar in Petri dishes and a few drops of a n-C_{12} - C_{18} fraction of hydrocarbon were put on to filter paper in the lid. Hydrocarbon-free control plates were also set up. Results were

read after 4 days incubation.

Growth on "orthodox" media. The ability of the organisms to grow on nutrient agar and yeast extract medium was tested by plating out on half strength Oxoid Bacteriological Peptone or 0.1% w/v Oxoid Yeast Extract, solidified with Oxoid No. 3 Agar. Results were read after 4 days.

Utilization of intermediates of gaseous hydrocarbon metabolism. Isolates were streaked on to basal salts medium in Petri dishes and a few drops of the substrate added to a piece of filter paper in the lid. Substrates tested in this way were the primary and secondary alcohols and methyl ketones. The acids were incorporated into the medium as the Na-salts (0.1% w/v). Substrate-free control plates were also set up. Results were read after 7 days.

Growth in liquid medium. Isolates were grown in liquid culture with the gas upon which they were isolated. Growth rates and culture appearance were visually observed over a period of 6-10 days.

Isolation of Non-clumping Mutants

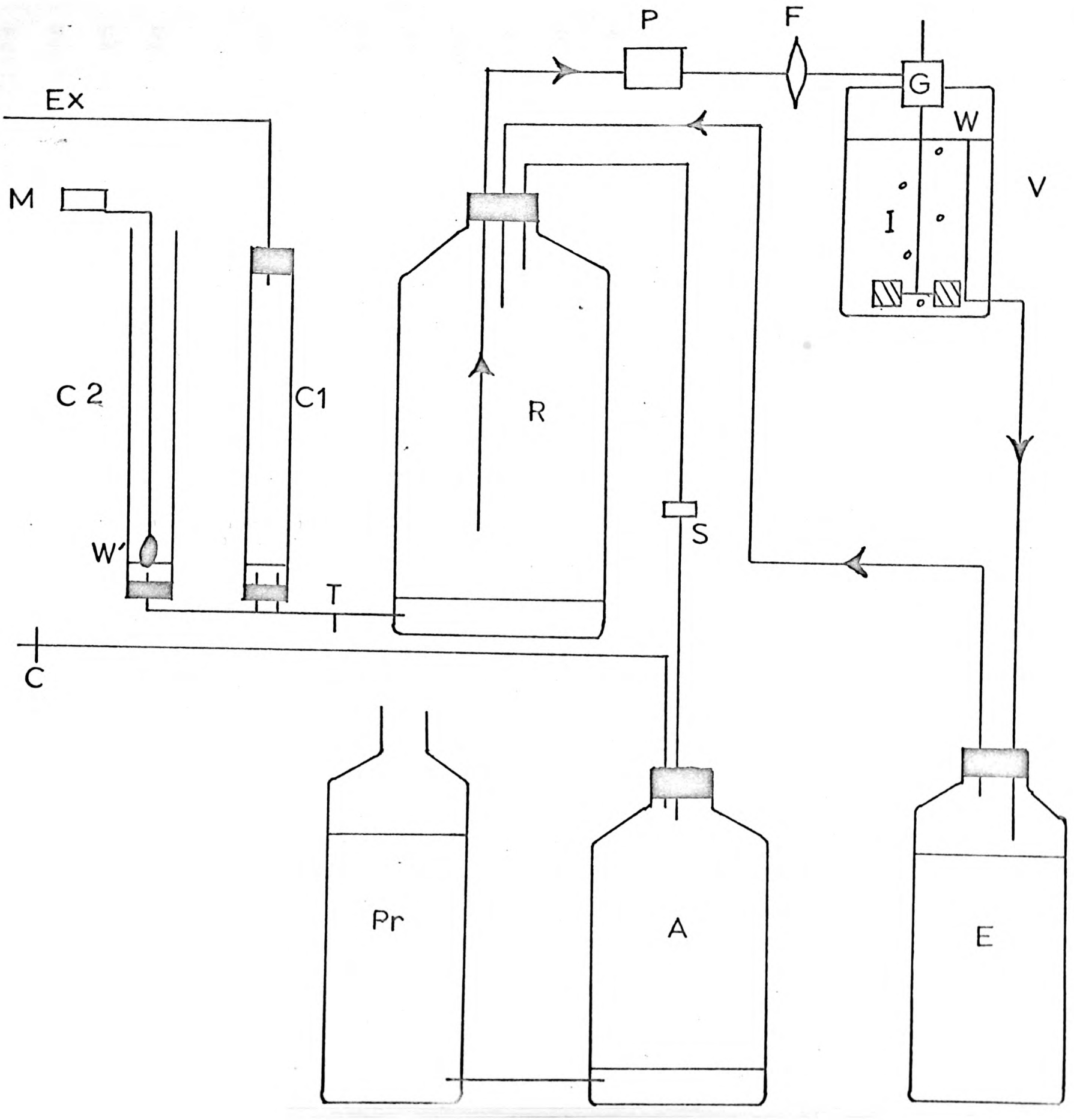
Ten ml. of a dense liquid culture was centrifuged in a 6 x $\frac{5}{8}$ in. test-tube for 10 minutes in a bench centrifuge. Microscopic examination was made of samples of the upper half of the clear supernate to ensure the presence of single cells only. A 1 ml. sample of this supernate was used as an inoculum for a second liquid culture to be grown on butane.

This culture was grown until a slight turbidity was visible to the eye, and then 1 ml. was used as an inoculum for a third culture. Inocula were similarly subcultured 2 more times. A series of 10-fold dilutions of the final culture was made in sterile medium and spread plates prepared, using 0.2 ml. samples of each dilution. These plates were incubated in a hydrocarbon atmosphere until they could be examined by a plate microscope for less rough colonies.

Slide-Culture Studies

Strains P1 and BG28 were grown on slide-cultures with a basal salts agar supplemented with 0.1% (w/v) Na-butyrate. In a second preparation, butane was used as a carbon and energy source. A loopful of inoculum was carefully spread over the agar surface and the preparations incubated in Petri dishes with a piece of moistened filter paper to prevent desiccation. Cultures were examined periodically over 24 hours by phase contrast microscopy.

Figure 26. Chemostat Design for Gaseous Hydrocarbon-Utilizing Bacteria



Key to Figure 26

1) Gas Circuit

- P Flow Inducer
- F Line Filter
- G Stirrer Gland
- I Impellor
- W Overflow Weir
- E Effluent Vessel
- R Gas Reservoir

2) Pressure Regulating Columns

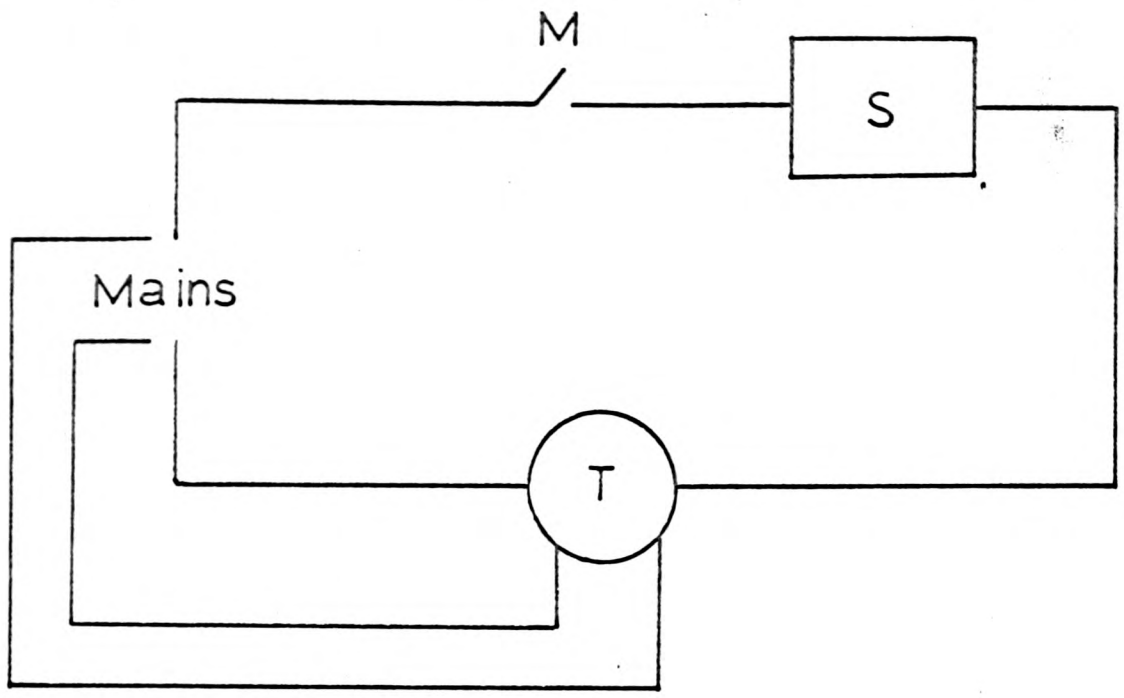
- C_1 Column 1
- C_2 Column 2
- W′ Weight
- T Tap
- M Microswitch
- Ex Exhaust

3) Gas Addition Vessels

- A Addition Reservoir
- Pr Pressure Vessel
- S Solenoid
- C Clip

Gas Flow

Figure 27. Circuit Diagram for Chemostat Gas Valve



Key

M Microswitch

S Solenoid Valve

T Timing Device

Continuous Culture Studies

The chemostat described in the section dealing with the continuous culture of yeast on liquid hydrocarbons was modified to accommodate a recycling atmosphere of gaseous hydrocarbon and air (Fig. 26). The system can be envisaged as existing in 3 integrated units, the gas circuit, the pressure regulating system and the gas addition vessels. (The circuit for the electrically operated components is shown in Fig. 27.)

In the gas circuit, a continuous flow of a mixture of equal volumes of air and hydrocarbon was passed through the impellor system of the growth vessel, by means of a Watson-Marlow Flow Inducer (P), delivering 550 ml. per minute. (For details of the construction of the impellor and gland see Fig. 11.) After the gas had been sparged through the growth vessel, it passed out via the overflow weir (W) into the effluent vessel (E), from which it was drawn back into the gas reservoir.

Attached to the gas reservoir were 2 pressure regulating columns, C1 and C2. When there was a pressure fall in the circulatory system, the water levels in C1 and C2 fell, exposing the balanced counter-weight (W), which caused the microswitch (M) to close and open the solenoid valve (S). Fresh gas could then enter the gas circuit reservoir (R). If for any reason the valve failed to close, the water would rise up column 2 to a certain level until the outlet in the gas

reservoir was exposed, and then excess gas would go to exhaust via column 2.

The system was filled with gas by displacing water from addition reservoir (A) to pressure reservoir (Pr). This was effected by opening clip (C) and passing gas under pressure from a cylinder into the addition reservoir. Water, completely filling gas reservoir (R), was displaced by gas from reservoir A through a tap (T). Sufficient water was left to operate the pressure columns.

The only part of the gas circuit which was sterilised was the section from line filter (F) to the outlet side of the effluent vessel.

As a safety precaution, a Venner Timer Unit was incorporated into the microswitch/solenoid circuit (Fig. 27). This ensured that the solenoid did not remain switched on indefinitely if a leak occurred in the circuit. Usually the timer setting was for 1 hr., thus if the sum total of the periods of the solenoid operating times exceeded this time, the valve was permanently switched off.

When the apparatus was assembled initially, there were virtually no leaks at the various connections. The main source of leakage was at the gland seal where the impellor shaft entered the vessel. Initially, loss of gas was about 700 ml. per hr., but this greatly increased during a run as the seal became worn. Although the gas mixture was not explosive as the hydrocarbon concentration was too high, an explosive gas

mixture may have been formed at the site of leakage by the impellor shaft, but the rotation of the drive shaft served to disperse the gas.

In later continuous culture enrichment experiments, the recycling system was by-passed, and a hydrocarbon/air mixture was passed directly through the growth vessel before going to exhaust. The hydrocarbon was withdrawn directly from a cylinder at a rate of 200 ml. per minute and mixed with an air flow of similar rate. As before, this gas mixture was filter sterilised before entering the vessel.

Enrichment for Shear-Resistant Isolates

An enrichment for shear-resistant isolates was set up in the continuous culture vessel. An atmosphere of technical grade ethane and air was passed through the apparatus, running in batch culture. The vessel was filled with medium, and twenty 1 ml. samples of mud and water were added. The chemostat was switched on, and run for 2 weeks. After this period, the contents were examined for ethane-utilizing micro-organisms.

Gas Liquid Chromatography Techniques

Qualitative and quantitative estimations of gaseous hydrocarbons; intermediates of gaseous hydrocarbon metabolism; oxygen and carbon dioxide were performed on a Pye Series 104 Chromatograph, using flame-ionisation or thermal-conductivity techniques. Substances for analysis were presented either in aqueous solution or the gaseous state. Gases were introduced either by syringe injection (1 ml. Gillette Scimitar disposable) or by means of a gas injection loop.

The thermal-conductivity detector (T.C.D.) was used to measure O_2 and CO_2. If the experiment also involved hydrocarbon estimation, these compounds were also measured on the T.C.D. Helium was chosen as the carrier gas since it permitted the safe use of high bridge currents and subsequently greater sensitivity was achieved. Reproducibility was good, errors being 0-5% in identical samples when measured on a Vitatron UR 400/2 M recorder and integrator. Gases could be measured down to a concentration of 0.5% (v/v) in a 0.5 ml. sample.

The flame-ionisation detector (F.I.D.) was used to measure organic solids or gases. Gaseous hydrocarbons could be measured at concentrations as low as 500 p.p.m. using a 0.5 ml. gas sample; and alcohols could be detected at concentrations of 0.002 mM/ml. solution. Normally a 20 μ l. sample of the solution was used. The error was usually of the order 0 - 5 or 6%. As with the T.C.D., a Vitatron recorder/integrator was

used for quantitative estimations. Oxygen-free nitrogen was used as a carrier gas.

Column Preparation

Initial attempts to detect C_2 - C_4 fatty acids in aqueous solution by the method of Rogosa and Love (1968) were unsuccessful. With untreated Phasepak Q, a stable complex involving water, the acid and the packing material was formed (Phase Separations Ltd., private communication). However, separation was achieved by 10% "trimer acid" treatment of the packing material. This was performed as follows. Five g. of "trimer acid" were dissolved in 200 ml. acetone and 50 g. Phasepak Q (Phase Separations Ltd., Queensferry, Flints) were added. The mixture was shaken gently (so as not to produce fines), and the acetone allowed to evaporate. The treated material was then dried overnight at 104° to remove the last traces of acetone.

Columns were packed by blocking one end with a glass-wool plug and attaching a vacuum pump. Material was introduced from the other end in small amounts and the column constantly vibrated to ensure uniform settling. Newly packed columns were equilibrated by heating at 120° for 2 days, whilst being purged with carrier gas flowing at 60 ml. per minute. It was found necessary to maintain a carrier gas flow of 40 ml./min. through equilibrated Phasepak Q columns during storage. Failure to do this caused "column bleed" during operation, thus

Table 13

Details of Gas/Liquid Chromatography Techniques

Detector	Column	Separating Material	Temperature	Carrier Flow Rate	Materials Separated
T.C.D. operating at 240 mA	Glass 1.5 m. by 6 mm.	Molecular Sieve 5A 60/80 mesh	75 - 80°	He 60 ml./min.	O_2 and N_2
"	"	Silica Gel 80/100 mesh	"	"	CO_2 and gaseous hydrocarbons
F.I.D.	"	10% Trimer acid treated Phasepak Q	130°	N_2 60 ml./min.	C_1 - C_4 n-alkanes
"	"	"	155 - 160°	"	C_2 - C_4 primary alcohols aldehydes fatty acids
"	"	Phasepak R	"	"	2-butanone and butyraldehyde

producing a high background signal. Molecular Sieve and Silica Gel columns were sealed when not in use. All columns possessed a long operating life when properly maintained. (See Table 13 for operating conditions for separation of the various compounds.)

Detection of Intermediates of Gaseous Hydrocarbon Metabolism

Strain P1 was grown on propane and butane and the culture fluid was analysed for intermediates. After 2-3 days incubation, the bacteria were spun down, leaving the spent medium, which was acidified by adding 2-3 drops concentrated HCl to 10 ml. medium. Samples (20 µl 1.) were analysed on the flame-ionisation detector using 10% (w/v) "Trimer" acid treated Phasepak Q and untreated Phasepak R to separate the mixture components.

Acetone, which was inseparable from propionaldehyde on either of the above column packing materials, was separated by the distillation method of Lukins (1962). Volumes of 4-5 l. of culture medium were distilled.

Products of ethane metabolism by strain BG28 were detected by flame-ionisation methods.

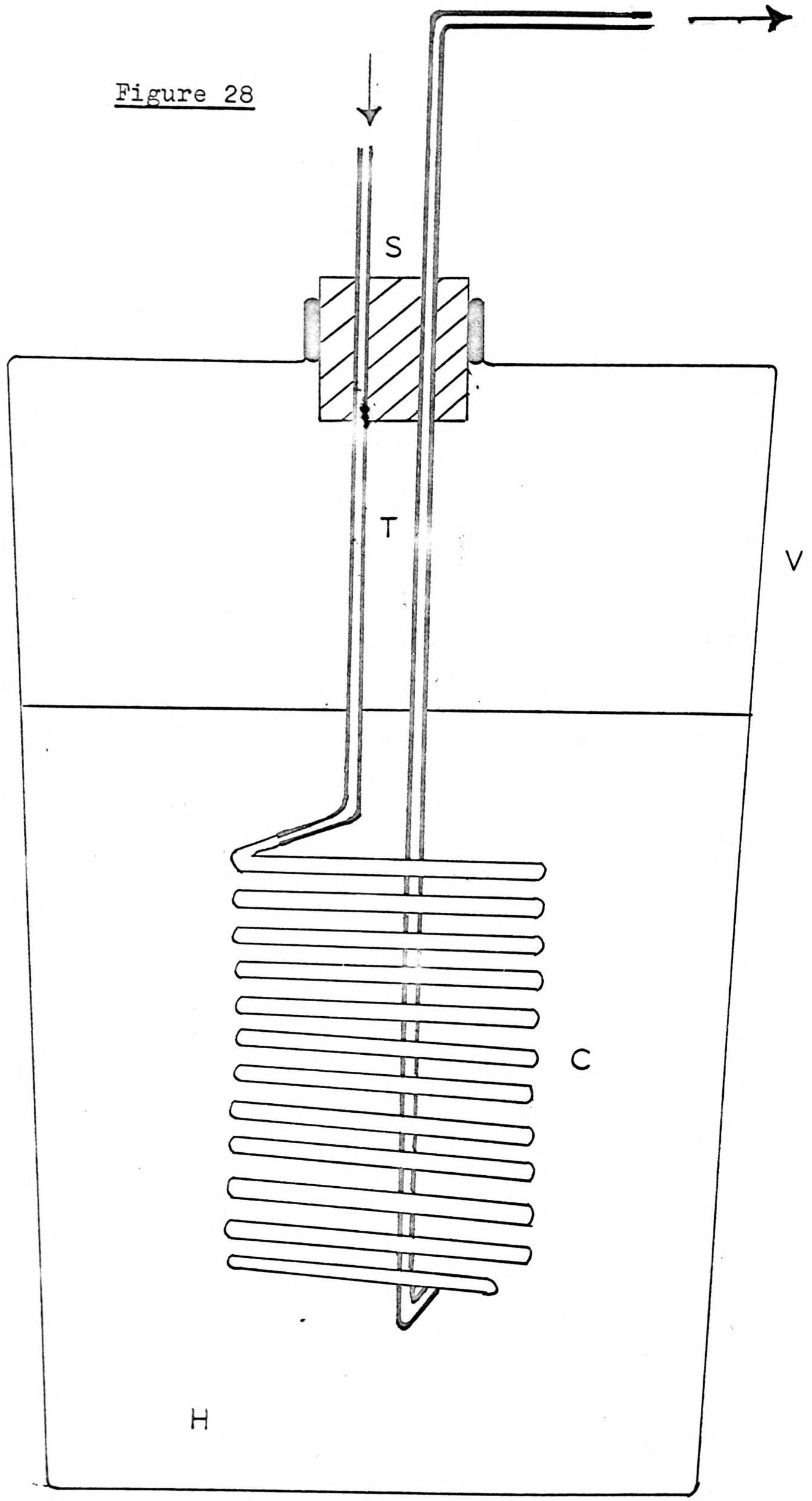
Measurement of Dissolved Hydrocarbons

Measurement of dissolved gases was performed by a modification of the method of Roberts and Shepherd (1968). PTFE tubing, permeable to the gases, was immersed in the solution. Gas diffused through the wall and into the tube, where it was transported by an inert carrier gas to a suitable detector, in the case of gaseous hydrocarbons - a flame-ionisation detector. The amount of gas in the carrier flow depended upon the rate of diffusion into the tubing, which was proportional to the concentration of dissolved gas outside.

Probe Construction

Four hundred cm. of PTFE tubing (Size No. STW 17, I.D. 1.3 mm., wall 0.15 mm.; Polypenco Ltd., Welwyn Garden City, Herts.) were coiled around a stainless steel wire support, 6 cm. in diameter. This support was constructed by welding eight 5.5 cm. cross-pieces of wire at 90^{o} to 2 parallel lengths of wire, forming a ladder-like structure 20 cm. long, with 2.5 cm. between each "rung". This structure was carefully bent to form a cylinder. The PTFE coil was attached to this structure by smearing the rungs with Araldite and then winding the coil around. Lengths of 1.58 mm. I.D. stainless steel pipes were fixed to each end of the coil, and the whole structure allowed to harden at room temperature for 2 days. The lengths of piping were led through a Suba seal in the lid of a Tupperware container (563-7) leaving lengths for

Figure 28



Detection Probe for Measurement of Dissolved Gaseous Hydrocarbons

Key to Figure 28

V Tupperware Vessel

S Suba Seal

T Stainless Steel Tubing

C PTFE Coil

H Aqueous Hydrocarbon Solution

⟶ Direction of Carrier Gas Flow

attachment to the chromatogram.

Measurement of Probe Sensitivity

Five hundred ml. of water were placed in a Tupperware container and 3-4 l. of hydrocarbon were bubbled through a glass tube inserted through the aperture in the container lid. The vessel was then sealed and shaken vigorously for 5 min. before being allowed to stand for 3-4 hours. After this period the lid was quickly removed and the modified lid with its probe attachment was fitted, completely immersing the PTFE coil (Fig. 28). Ten ml. samples of the carrier gas flow (50 ml. per min.) were taken at regular intervals using the gas sampling loop. This procedure was performed for all 4 gaseous hydrocarbons. With methane, the experiment was repeated using a carrier flow rate of 30 ml. per min. to observe any effect reduction of flow rate may have on sensitivity.

The rate of response to an instantaneous reduction in concentration of dissolved gas was measured by noting the time required for the readings to reach a constant value. This was achieved by diluting the dissolved methane concentration with 250 ml. water and taking readings. A carrier flow rate of 50 ml. per min. was used.

Measurement of Gas Transfer Rates

Twenty ml. volumes of each gas were injected into a

250 ml. sealed flask containing 50 ml. water. The flask was vigorously agitated at room temperature for 30 minutes to ensure saturation of the water with each gas. A 20 ml. sample of water was withdrawn from the inverted flask and carefully injected into another sealed flask, also held in the inverted position. This procedure was followed to prevent splashing of water causing a rapid transfer of gas into the air before readings could be taken. Samples of air (0.5 ml.) were withdrawn at 3 min. intervals and the gases measured using flame-ionisation detection.

Growth Rate Studies

The growth rates of strains P1 and BG28 on gaseous hydrocarbons at different partial pressures were measured as follows. Mixtures of a hydrocarbon and oxygen, varying from 10-90% (v/v) hydrocarbon, were made by the downward displacement of water from a sealed medical flat. The pressure inside was slightly above atmospheric pressure. Samples (0.2 ml.) of hydrocarbon-adapted cultures were inoculated into a series of 6 x $\frac{5}{8}$ in. test-tubes, containing 5 ml. nitrate basal salts medium. The protruding piece of cotton-wool plug was cut off, leaving a length of 1.5 cm. blocking the tube neck. This remaining piece was pushed 1 cm. down the tube to ensure no fibres were left to break the air-tight seal, when a Suba seal was fitted. Tubes were evacuated for 10-15 seconds by thrusting a thin hypodermic needle through the seal and attaching to an oil

vacuum pump. The prepared gas mixtures were added by joining the medical flat to the evacuated tube by a 14 cm. piece of thin PTFE tubing with hypodermic needles attached to each end. When the connecting tube was attached to the gas mixture, a delay of 2-3 seconds was allowed before the other end was attached to the test-tube. This was to allow air in the PTFE tube to be blown out by the gas mixture. Each gas mixture was used to supply two test-tubes. Cultures were incubated at 30° in a position as near horizontal as possible. Before optical density readings were taken (Eel Nephelometer, OR 2 light filter), the cultures were agitated for 4-5 sec. with a Fisons Whirlimixer. Optical densities were measured when the readings exceeded 20 units until values of 80-90 were obtained. THE EXPERIMENT WAS PERFORMED TWICE.

Temperature Optimum

Strains P1 and BG28 were grown at their optimum hydrocarbon/oxygen partial pressures in sealed test-tubes prepared as above. Growth rates were studied at 22, 25, 30, 34 and 37° on nitrate and ammonium basal salts media. THE EXPERIMENT WAS REPEATED.

Growth Rates with Mixtures of Hydrocarbons

Strains P1 and BG28 were grown in atmospheres of 50% v/v oxygen and a mixture of two or all three hydrocarbons. When two hydrocarbons were present, each comprised 25% of the partial pressure, and when three were present, each comprised 16-17%. Experimental procedure and measurement of growth

rates were as described above. THE EXPERIMENT WAS REPEATED.

Hydrocarbon Preferences

The preferential utilization of a hydrocarbon in the presence of an equimolar amount of one or two other hydrocarbons was measured as follows. Twenty ml. of each gas were injected into a culture of Pl or BG28 and incubated overnight. Gas uptake rates were measured by periodically withdrawing 0.5 ml. samples and analysing with a flame-ionisation detector. THE EXPERIMENT WAS REPEATED.

Adaption Studies

Strain P1 was grown on propane, butane and possible intermediates in the metabolism of these gases. Intermediate substrates tested were 1-propanol and 1-butanol; 2-propanol and 2-butanol; 2-propanone and 2-butanone; 1-hydroxy-2-propanone (acetol); propionic and butyric acids. All substrates were supplied in basal salts medium at 0.05% (v/v or w/v). Only the acids, supplied as the Na-salts, were sterilised before addition to the medium. Gaseous hydrocarbons were supplied in 50 ml. volumes. All growth flasks, except those containing the acid substrates, were sealed during incubation to prevent volatilisation of substrate.

After 4-10 days incubation, cells were separated from their growth media and resuspended in salts solution. Spent growth media was stored at 4^{o} for intermediate analysis.

Adaption to a particular substrate was determined by measuring O_2 uptake rate on a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co. Inc., Ohio). Three ml. samples of the cell suspensions were placed in the instrument's O_2-uptake measurement chambers, and 0.3 ml. of substrate (at 0.1 M concentration) added. The suspension was allowed to equilibrate for 3 minutes before the O_2 electrode was inserted. For gaseous substrates, a mixture of air and hydrocarbon was bubbled through the suspension for 1-2 minutes before electrode insertion. Oxygen uptake rates were recorded and expressed as a percentage increase of the endogenous rate. Endogenous rates were measured after each series of 4 samples.

Manometric and Growth Yield Studies

Molar oxygen uptake and carbon dioxide production per mole of substrate was measured on the following substrates, propane and butane; 1-propanol and 1-butanol; 2-propanone and 2-butanone. Dry weight yields were also determined. The organism employed was strain P1 growing in batch culture.

Ten per cent (v/v) solutions of the non-gaseous substrates were made by adding 10 ml. of unsterilised substrate to 90 ml. sterile water. Volumes of these dilutions containing 1 m. M substrate were added to 50 ml. medium, which were inoculated with hydrocarbon-grown cells and sealed. Fifty ml. hydrocarbon were added to other flasks. Five flasks were set up for each substrate.

After 7-14 days incubation, the oxygen and carbon dioxide concentration in the flasks was measured using the thermal-conductivity detector. A sample size of 0.5 ml. was used. When hydrocarbon was the substrate, this too was measured on this detector. Dry weights were measured by centrifuging down the cells, washing once in distilled water and drying for 2-3 days at 104^{o}. Weighing, to 0.1 mg., was performed on an Oertling single-pan balance. Unused substrate was measured by injecting 20 μ l. samples of supernate into the flame-ionisation detector.

Mean results were expressed as moles O_2 used, or CO_2 produced, per mole of substrate. Cell yields were expressed as percentages.

Lipid Fractionation

Strain P1 was grown in batch culture on propane, butane and the corresponding primary alcohols (0.1% v/v). Organisms were harvested, washed once in distilled water, and freeze-dried in an Edwards Model 30 P2 Centrifugal Freeze Dryer. Bacterial samples (0.5 - 0.7 g.) were weighed and added to 50 ml. of a 2:1 chloroform/methanol mixture for overnight lipid extraction at 55°. The cells were separated from the lipid solvent by filtration through Whatman Number 1 filter paper, and the chloroform/methanol mixture removed by rotary evaporation. The glyco-lipid fraction was removed by dissolving in 100 ml. di-ethyl ether and filtering. The residue, containing waxes and poly β -hydroxybutyrate, was first treated with hot acetone, and then decanted whilst still warm. On cooling the waxes precipitated. The remaining fraction was almost entirely soluble in chloroform. The chloroform-soluble, and hot acetone-soluble fractions were pooled, since separately there was insufficient material to give significant dry weights. The two fractions, the ether-soluble and ETHER-insoluble, were evaporated to dryness in a rotary evaporator and stored overnight in the freeze dryer before weighing. Results were expressed as a percentage of the total cell dry-weight.

Pristane Studies

Five ml. of a hydrocarbon-adapted culture of strain P1 was used to inoculate flasks containing 2 ml. of pristane in

50 ml. salts solution. Fifty ml. of propane or butane were added as substrate. Control flasks containing no pristane were also set up. When growth in the control flasks corresponded to a reading of 20-30 units on a nepholometer, growth rates were studied by following the rate of uptake of the propane or butane in the flasks. This was performed by the periodic removal of 0.5 ml. samples of the gaseous atmosphere and quantitatively estimating the hydrocarbon content on the flame-ionisation detector.

Gas Uptake by Non-proliferating Cells

Ten ml. volumes of hydrocarbon-grown P1 cells were centrifuged in a 6 x $\frac{5}{8}$ inch test-tubes and the spent medium poured off. Cells were killed by heating the pellet for 2 minutes at 100° or by adding 2-3 drops of formalin to the culture before centrifugation.

An atmosphere containing equimolar amounts of C_1 - C_4 n-alkanes was prepared by injecting 20 ml. volumes of each gas into a sealed flask. This gas mixture was added to the treated cells by injecting 1 ml. into the test-tubes sealed with Suba seals. A control tube was set up, containing 0.1 ml. water to compensate for any hydrocarbon lost from the gaseous phase by dissolving in the water in the cell pellets. Any hydrocarbon uptake by the cellular lipid was measured by removing 0.5 ml. of the atmosphere and analysing the hydrocarbon content by flame-ionisation detection. Samples were

taken over a period of 3 hours with the cells kept at room temperature.

Gas Uptake by Freeze-dried Cells

Freeze-dried samples (0.1 g.) of P1 cells grown on propane, butane, 1-propanol and 1-butanol were placed in test-tubes and the uptake of gaseous hydrocarbons by cellular lipids was measured by the same technique as described above. The control containing water was omitted.

Electron Microscopy

The techniques used in the preparation of strain P1 for electron microscopy were those used by Davies and Whittenbury (1969).

Table 14

Colony Morphology and Staining Properties of Isolates

Isolate Group	Gram Stain	Acid-fast Stain	Capsule	Motility	Colony Diameter (mm)	Colony 5-7 days	Colour 12-14 days	Colony Texture	Colony Morphology
A	+ & -	+	-	+	1	Pale yellow	Yellow	Soft	"Draughtsman"
B	-	-	-	+	3 - 4	Pale brown	Brown	"	Flat, spreading
C	+ & -	+	-	-	1	Bright yellow	Bright yellow	"	"Draughtsman"
D	-	-	-	+	< 1	Pale yellow	Yellow	"	Domed, entire
E	+	+	-	-	1 - 2	White	Pinkish	Dry	Rough, irregular
F	-	-	+	-	1 - 2	White	White	Very mucoid	Domed, entire
G	+	+	-	-	1 - 2	Pale yellow	Dirty yellow	"	" "

Table 15

Cellular Morphology of Isolates

Isolate Group	Number of Isolates	Size (μ)	Description
A	6	2	Cocco-bacilli, single or pairs
B	3	2 - 3	Thin, curved or straight rods. Single
C	2	2 - 3	Cocco-bacilli, single, pairs or clumps
D	1	1 - 1.5	Small single rods
E	3	2 - 3	Cocco-bacilli, single, chains or clumps
F	2	2	Cocco-bacilli, single or pairs
G	1	2 - 3	Rods, single or pairs

Table 16

General Growth Properties

Isolate Group	H_2	C_1	C_2	C_3	C_4	*n*-alkanes Liquid C_{12} - C_{18}	NH_4^+ & NO_3^-	Nutrient Agar
A	-	-	+	+	+	-	+	+
B	-	-	+	+	+	-	+	+
C	-	-	-	+	+	+	+	+
D	-	-	+	+	+	-	+	+
E	-	-	+ or -	+	+	+	+	+
F	-	-	-	+	+	+	+	+
G	-	-	-	+	+	+	+	+

Table 17

Properties of Industrial Significance

Isolate Group	Growth at Elevated Temperatures				Urea Utilization	Growth on Gaseous Hydrocarbon in Liquid Culture
	33	37	40	45		
A	-	-	-	-	-	Very granular
B	-	-	-	-	-	Poor
C	-	-	-	-	-	Granular
D	-	-	-	-	-	Poor
E	+	+	-	-	-	Good, granular
F	-	-	-	-	-	Poor
G	-	-	-	-	-	Good

RESULTS AND DISCUSSION

Growth Techniques

Desiccators were convenient containers for cultures growing in hydrocarbon atmospheres. The vessels were periodically dried and swabbed with ethanol to discourage the growth of mould.

Enrichment and Isolation

Organisms oxidising ethane, propane and butane were encountered less frequently than those utilizing methane; only 20% of the enrichments yielded C_2 - C_4 n-alkane-oxidising organisms, whereas 80-90% yielded methane utilizers (Whittenbury, private communication). This difference is probably because of the scarcity of C_2 - C_4 n-alkanes in the environment.

In the early stages of isolation, it was necessary to pick off colonies as soon as possible to prevent their being consumed by amoebae, or lysed by myxobacteria.

Morphology of Isolates

(See Tables 14 and 15.)

Selection of Isolates for Further Studies

All isolates were screened in a series of biochemical and physiological tests to select the most suitable strains for further investigations (Tables 16 and 17). Two of the

isolates of group E, P1 and BG28, were chosen since they grew most readily and had a wide range of properties.

Classification

Studies of slide cultures of strains P1 and BG28, growing on butane and on butyrate, revealed that they formed a mycelium, which fragmented into separate cells after about 12 hr. These isolates were identified as Nocardia species. The utilization of gaseous hydrocarbons by Nocardia has been reported previously by Davis (1964b).

Features of Industrial Significance

The purpose of this report was to examine generally the physiological and biochemical characteristics of hydrocarbon-utilizing bacteria. The results in Table 17 indicate that features likely to be of industrial significance were not normally typical of these isolates. It would be necessary to select for such abilities as growth at elevated temperatures and urea utilization, at the enrichment stage.

These properties would be particularly desirable for industrial application, since the ability to grow at high temperatures would minimise the expense of heat dissipating equipment; and urea utilization would permit the use of this cheap and easily synthesised nitrogen source.

General Observations of Isolates' Properties

(See Table 16.)

None of the isolates grew on methane, which suggested that previous reports of organisms growing on methane, and on other gaseous n-alkanes, were due to impure gases being employed. Also, Whittenbury reported the failure of methane-utilizing bacteria to grow on other gaseous hydrocarbons (private communication).

The property of hydrogen autotrophy, a feature of bacteria isolated on hydrocarbons, was found to be lacking in all isolates. It may not be widespread as Lukins (1962) supposed.

The absence of hydrocarbon specificity in all isolates indicated that Mycobacterium paraffinicum had not been isolated. This would further suggest that none of the soil samples had been taken in areas of petroleum seepage, thus accounting for the low frequency of isolation of C_2 - C_4 n-alkane-utilizing organisms.

Isolation of Non-clumping Mutants

As the granular nature of the growth of strains P1 and BG28 in liquid medium made measurements of growth rates by turbidimetric methods impossible, mutants were selected which did not clump. Selection involved differential centrifugation to remove cell aggregates, and subculturing the free organisms in the supernate. Mutants were recognised by their smooth colony forms.

The mutants obtained were stable, not reverting to the rough colony form of the wild type, and growth in liquid culture remained uniformly turbid. Microscopic examination of such cultures showed most cells growing singly or in pairs. These variants were used in all later experiments on gaseous n-alkane utilization.

Behaviour of Organisms after Inoculation

When a loopful of bacteria, taken from an agar slope, was used as an inoculum for liquid culture, a surface tension effect was observed, the cells spreading rapidly across the liquid as a thin film. (A similar observation was made by Dworkin and Foster (1958) with hydrocarbon-grown Mycobacterium isolates.)

Agitation of the culture produced a suspension of uniform turbidity, which disappeared after 30 minutes, leaving a granular suspension. These few flaky particles persisted, but as growth proceeded, the culture again assumed uniform density. Microscopic examination of the culture showed macroscopic cell aggregates, clumps of 5-6 cells laterally adhering to each other, and many single cells. Presumably, this appearance was caused by the hydrophobic effect of the cell-wall lipid, which caused the bacteria of the inoculum to clump, and even to form large granules. As growth proceeded, a wetting agent appears to have been produced which prevented a similar aggregation occurring with the newly formed single cells, thus resulting

Table 18

Growth Substrates of Non-hydrocarbon Utilizing Mutants of Strains P1 and BG28

Substrate	Organism			
	P1	P1 mutant	BG28	BG28 mutant
Ethane	-	-	++	-
Ethanol	+++	+	+++	+
Acetate	++	++	++	++
Propane	+++	-	+++	-
1-propanol	+	+	++	+
2-propanol	++	+	++	+
2-propanone	+++	+	+++	+
Propionate	++	++	++	++
Butane	+++	-	+++	-
1-butanol	+	+	+	+
2-butanol	+	+	+	+
2-butanone	+++	+	+++	+
Butyrate	+++	++	++	++

\- no growth

\+ slight growth

++ growth

+++ good growth

finally in growth of uniform turbidity. Transfer of such a culture to fresh medium, or the addition of fresh medium to the culture, resulted in aggregates being reformed. Addition of Tween 80 or Silicone Antifoam Emulsion (0.01% v/v) prevented these aggregates forming, but inhibited growth.

The granulating behaviour of inocula meant that cultures had to be quite well grown if optical density readings were to be meaningful. (Usually readings were taken when the optical density exceeded a value of 0.25 - 0.30 measured at 540 nm. on a Unicam 500 Spectrophotometer; or was greater than a reading of 20 on an Eel Nepholometer.) As a result of this difficulty, growth rate studies could not be performed for periods longer than those corresponding to two doublings in optical density, lest some nutrient became lacking.

Spontaneous Occurrence of Non-hydrocarbon-Utilizing Mutants

When stock cultures of P1 and BG28 were periodically checked for purity and grown on gaseous hydrocarbons, two colony types appeared. One was the normal large rough colony of the isolate when growing on gases; the other was small and flat, having the appearance of the isolate when grown on agar without hydrocarbon. When this variant was grown on non-hydrocarbon substrates, it was identical to the parent strain.

This mutant grew on intermediates of gaseous hydrocarbons but only extremely slowly on the gases themselves (Table 18). This slight utilization of the gases was only obvious after

comparison with hydrocarbon-free controls after 2 weeks incubation. It would appear that only the hydrocarbon oxygenase system was affected by this mutation, and even then the blockage was incomplete.

Purification of the parent strain, and subsequent subculture, showed the spontaneous production of this mutant. Subculturing on ammonium medium increased the frequence at which the mutation occurred.

A similar type of mutation could account for the death of isolates comprising groups A and F. After 2-3 subcultures on gaseous hydrocarbons, all these isolates gradually died off. A similar total loss of viability on hydrocarbons has been reported by Dworkin and Foster (1958).

Continuous-Culture Studies

An attempt was made to grow strain BG28 on ethane in continuous culture. An inoculum (5% v/v) was injected into the growth vessel and the apparatus was run with a recycling gas atmosphere for 3 days. At the end of this period the inoculum had failed to grow, and, since no pH or temperature fluctuations had occurred, it was assumed that the inoculum was non-viable. The experiment was repeated with a 40-50% (v/v) inoculum. When the impellor was switched on the appearance of the culture changed from a cloudy white to a pale grey colour, accompanied by foaming and the smell of cysteine. Microscopic examination showed complete lysis of cells. The previously described high shearing forces, which had stripped the yeast cells of liquid hydrocarbon droplets, was also responsible for smashing these bacteria.

These forces seemed to affect the Nocardia isolates in particular, since P1 was similarly ruptured but an isolate of group D showed no lysis after 10 minutes stirring. Methane-utilizing organisms were unaffected and grew well in the system (Phillips, private communication).

Since no other isolates grew as well as these Nocardia, an enrichment was set up within the vessel to select hydrocarbon-utilizing organisms capable of withstanding the shearing forces. The apparatus was run for 2 weeks in batch culture with an uncycled ethane/air atmosphere passing through. Examination of the medium showed few cells and no hydrocarbon-

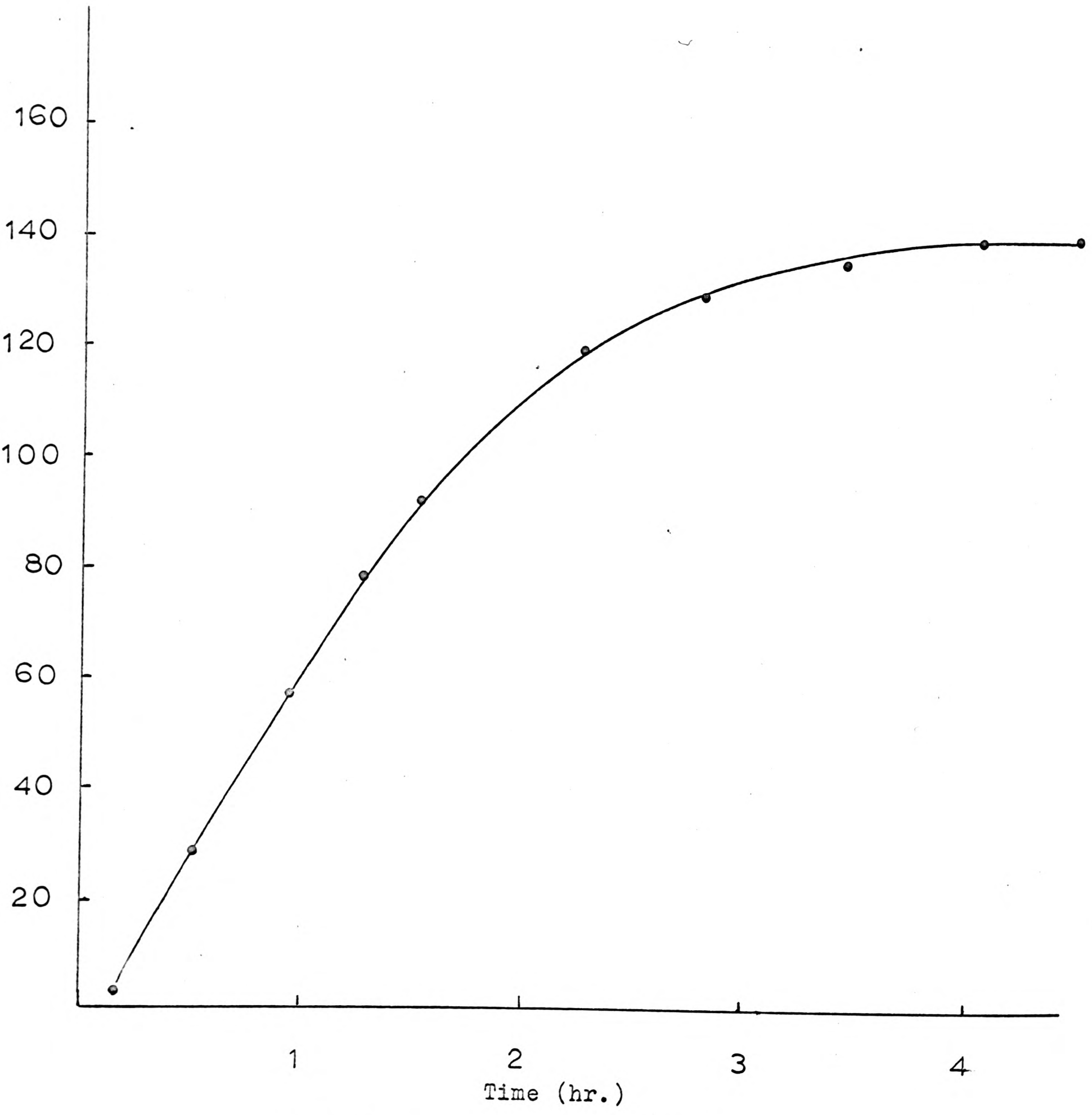
utilizing isolate could be obtained. Attached to areas inside the vessel was bacterial growth, which, after purification, yielded an ethane-utilizing organism similar to the rough wild types of P1 and BG28. This isolate seemed unable to survive in the vessel unless attached to some substratum. As a result of these findings it was decided to abandon continuous-culture studies in this type of vessel.

Measurement of Dissolved Hydrocarbon

A modification of the method used by Roberts and Shepherd (1968) to measure dissolved O_2, was used to measure dissolved gaseous n-alkanes (C_1 - C_4). A permeable coil of poly tetrafluoro-ethylene (P.T.F.E.) tubing was immersed in the aqueous solution and carrier gas flowed through (Fig. 28). Dissolved hydrocarbon diffused through the tubing wall and entered the carrier gas, by which it was transported to a flame-ionisation detector. The detector's response was directly related to the concentration of gas in the carrier which, in turn, depended upon the concentration of dissolved gas and the rate of transfer through the P.T.F.E.

The coil's sensitivity was estimated by measuring the time taken to respond to a change in dissolved gas concentration. In one case the coil was suddenly immersed in a gas solution and the time taken for the detector to register a constant value was measured. In a second type of experiment, the coil was equilibrated at a particular gas concentration and then the

Figure 29



Probe Response to Dissolved Ethane

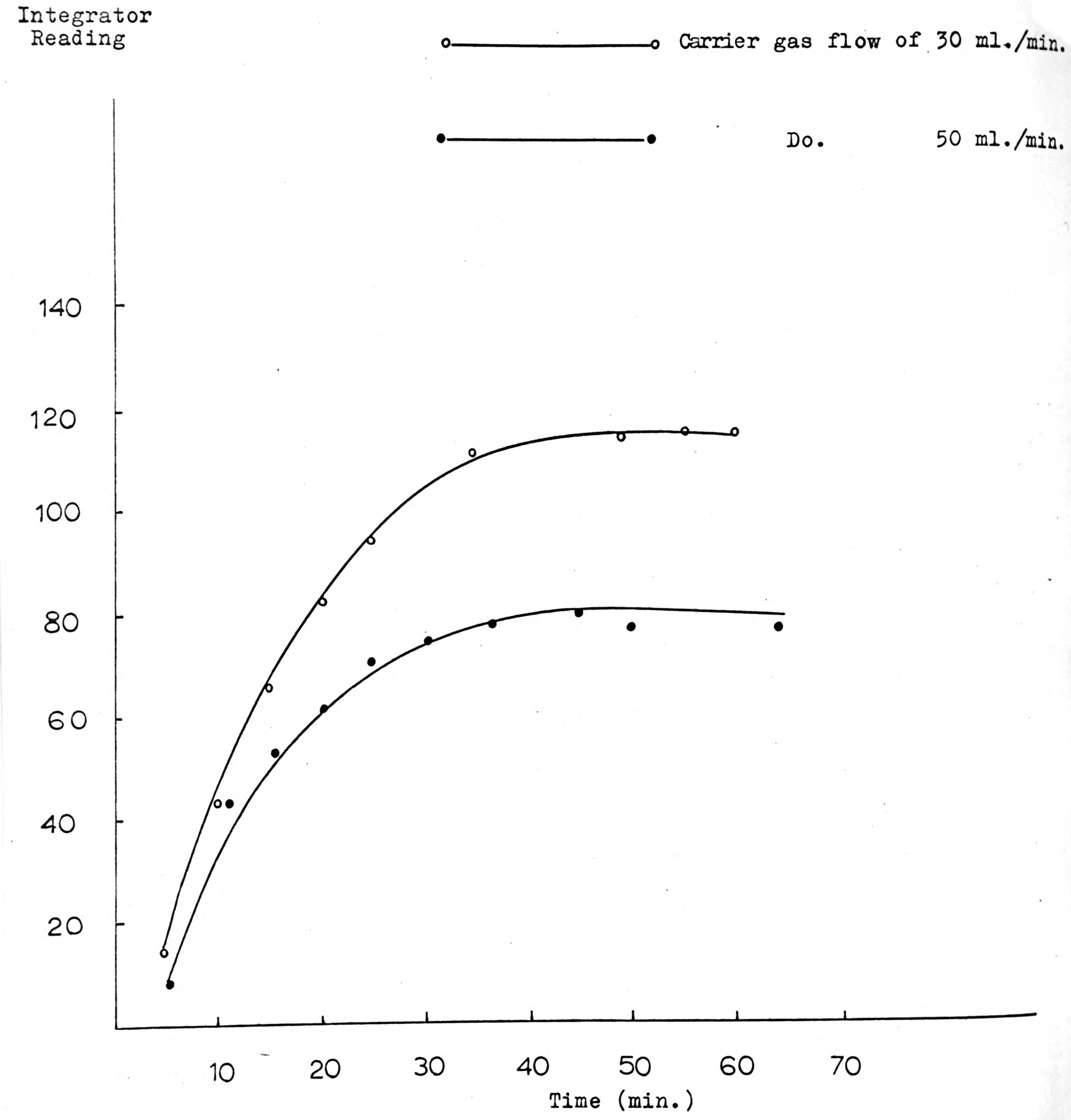
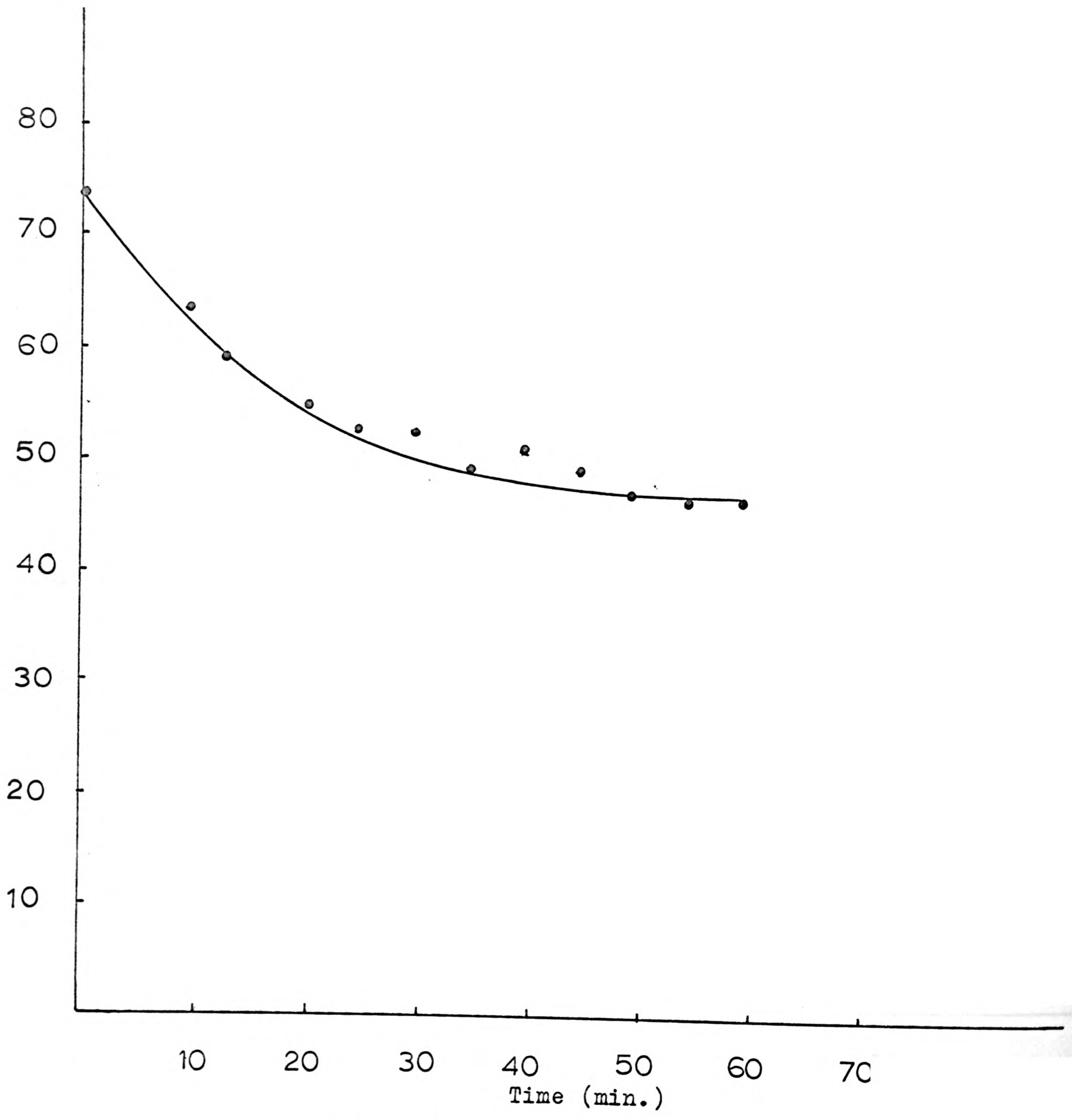


Figure 30. Probe Response to Dissolved Methane

Figure 31



Probe Response to Dissolved Methane

concentration was reduced by one third by diluting with water. As before, the time taken for the readings to stabilise was noted. This experiment was performed with methane only.

The response times of propane and butane were infinite, i.e. the gases failed to pass through the P.T.F.E. coil, or did so at a very slow rate. Ethane was found to have a response time of 3.5 - 4 hours (Fig. 29). However, methane had a response time of 35-40 minutes, which was unaffected by reducing the carrier gas flow rate to 30 ml./minute (Figs. 30 and 31). The concentration of methane in the carrier flow was increased, but it was insufficient to affect the rapid functioning of the transfer gradient, which might have decreased the coil's sensitivity.

The sensitivity and rapidity of response of this monitoring system permitted its application in the chemostat for measuring dissolved methane concentration (Phillips, private communication). The system could be further modified to become ultra-sensitive and to provide a continuous record of dissolved methane concentration, if the separating column were removed from the chromatography apparatus, and the methane concentration in the carrier gas was measured as a standing signal. Removal of the column would allow the detector to operate at more sensitive levels, and also dispense with the necessity of periodic sampling. Dissolved O_2 could also be simultaneously measured by such a system if electron-capture detection techniques were employed. If the concentration of

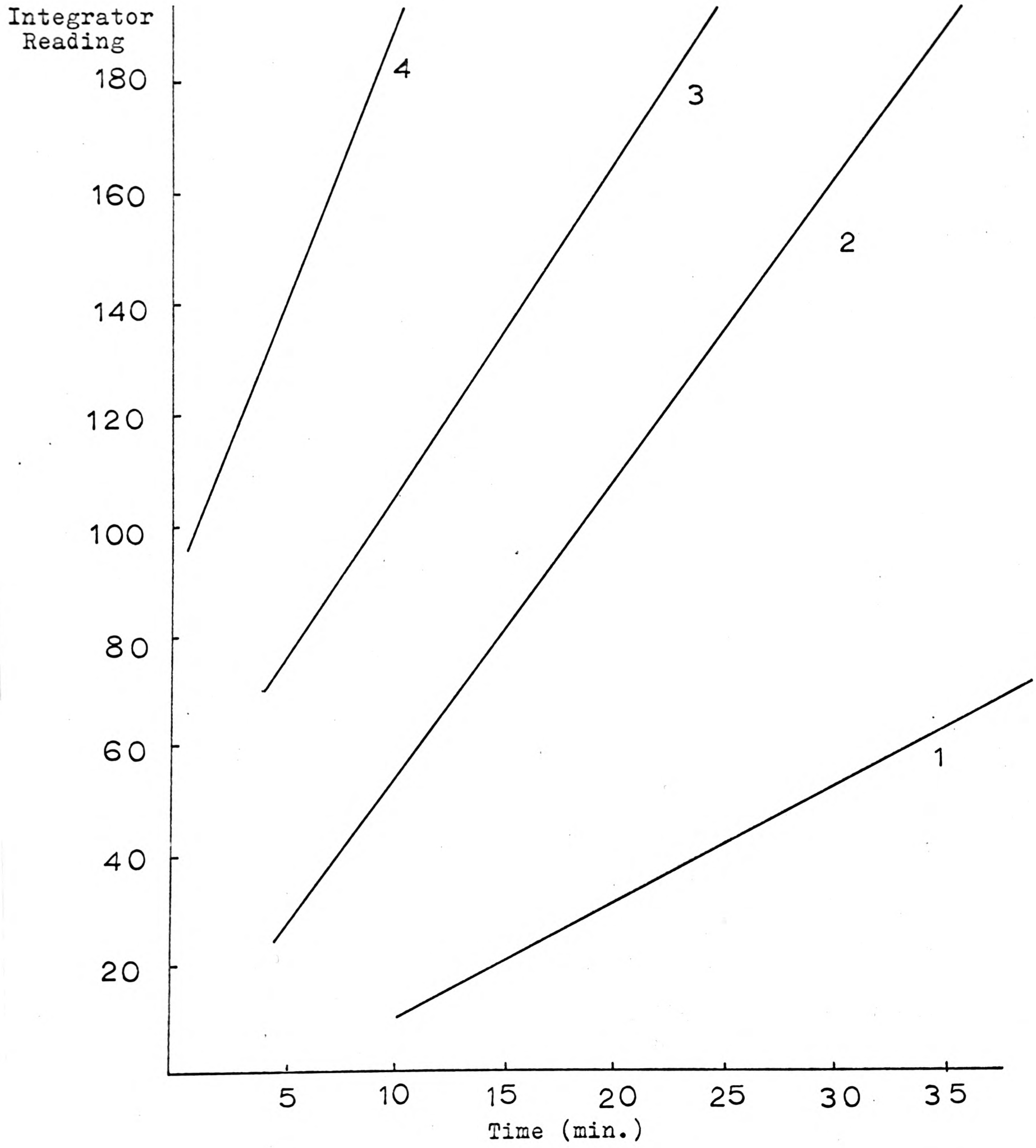


Figure 32. Hydrocarbon Transfer Rates

1. Methane
2. Ethane
3. Propane
4. Butane

O_2 in the carrier was sufficiently high, it may be possible to measure it by a thermal-conductivity detector.

The monitoring of the other gaseous hydrocarbons might be possible if a different type of tubing with a sufficiently thin wall could be obtained, which would permit rapid gas transfer.

Measurement of Gas Transfer Rates

An investigation to measure the transfer rates of gaseous hydrocarbons was carried out. It was thought likely that the availability of a gaseous substrate to bacteria in a dense culture would depend largely upon the rate at which the hydrocarbon could be transferred from the gaseous phase to aqueous solution (Mueller, 1969). Since rate coefficients depend upon the parameters of temperature, concentration gradient and surface area (Elsworth, Williams and Harris-Smith, 1957), measurements of rates of all gases were made simultaneously in the same vessel. Instead of measuring transfer rates from the gaseous phase into solution, the easier, reverse process was measured. This was achieved by shaking a volume of water with an atmosphere of equal partial pressures of the gases, and then transferring the gas solution by syringe to a second sealed vessel. Samples of the atmosphere were taken from this second vessel and the rates of transfer of the hydrocarbon to the air was measured by flame-ionisation detection (Fig. 32).

Since equimolar amounts of different gases do not ionise

Table 19

Ratio of Transfer Times of Equimolar Samples of C_1 - C_4 n-alkanes

n-alkane	Integrator Response to Equimolar Gas Samples	Ratio of Transfer Times of Equimolar Samples
Methane	43	1.65
Ethane	69	1.0
Propane	100	1.24
Butane	142	1.14

Table 20

Ratio of Dissolved Concentration of C_1 - C_4 n-alkanes at Equal Partial Pressures

n-alkane	ml. Gas Dissolved in 100 ml. H_2O at 25° under 1 atmosphere pressure	Ratios of Dissolved Concentrations
Methane	9	1.92
Ethane	4.7	1.0
Propane	6.5	1.38
Butane	15	3.18

to the same degree, the amount of ionisation (measured by a numerical integrator reading) of equimolar amounts of all gas was calculated and expressed as ratios (Table 19). From Figure 32 the times taken for these particular integrator values of hydrocarbons to be transferred were calculated, and these times expressed as ratios (Table 19). The relative transfer rates were found not to increase with carbon number. Methane had the slowest transfer rate, and ethane was the most rapid although ethane, propane and butane transfer rates were similar.

In order to have obtained a closer estimate of the relative rates, it would have been necessary to have all gases dissolved at the same concentration, thus having identical concentration gradients between the water and gaseous phase. However, only an approximation towards this ideal could be achieved, by initially dissolving the gases under equal partial pressures. (The ratio of the dissolved concentrations is shown in Table 20.) There would appear to be little correlation between dissolved concentration and transfer rate, since ethane, which has the fastest transfer rate, was dissolved at the lowest concentration, and also butane, with the highest dissolved concentration, was the second most rapidly transferred gas.

It seems that the differences between concentration gradients had little effect on the transfer rates. Probably the rate-governing step in gas transfer is the fluid diffusion

coefficient. The importance of transfer rate in its effect upon growth is discussed in the section dealing with industrial applications.

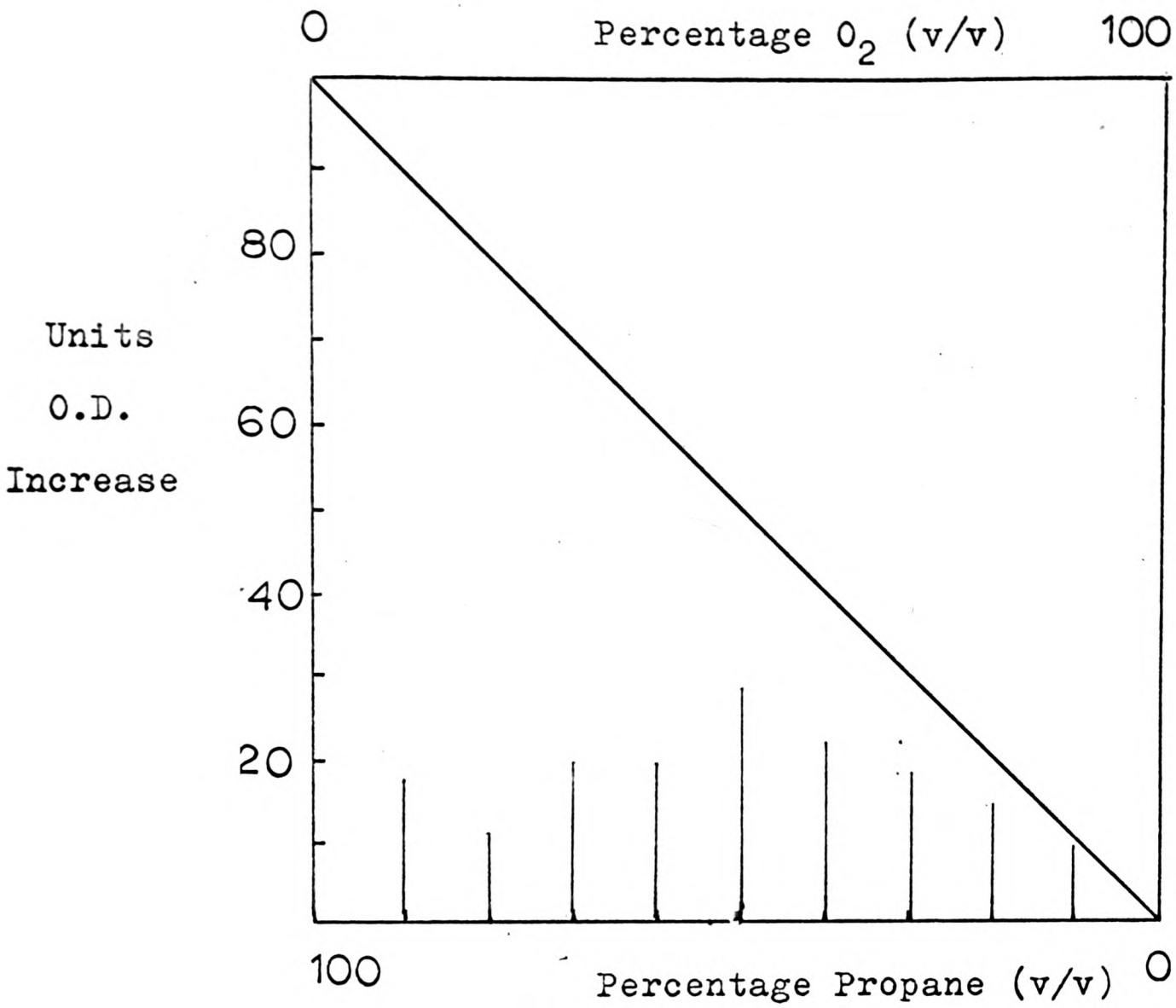


Figure 33

Strain P1

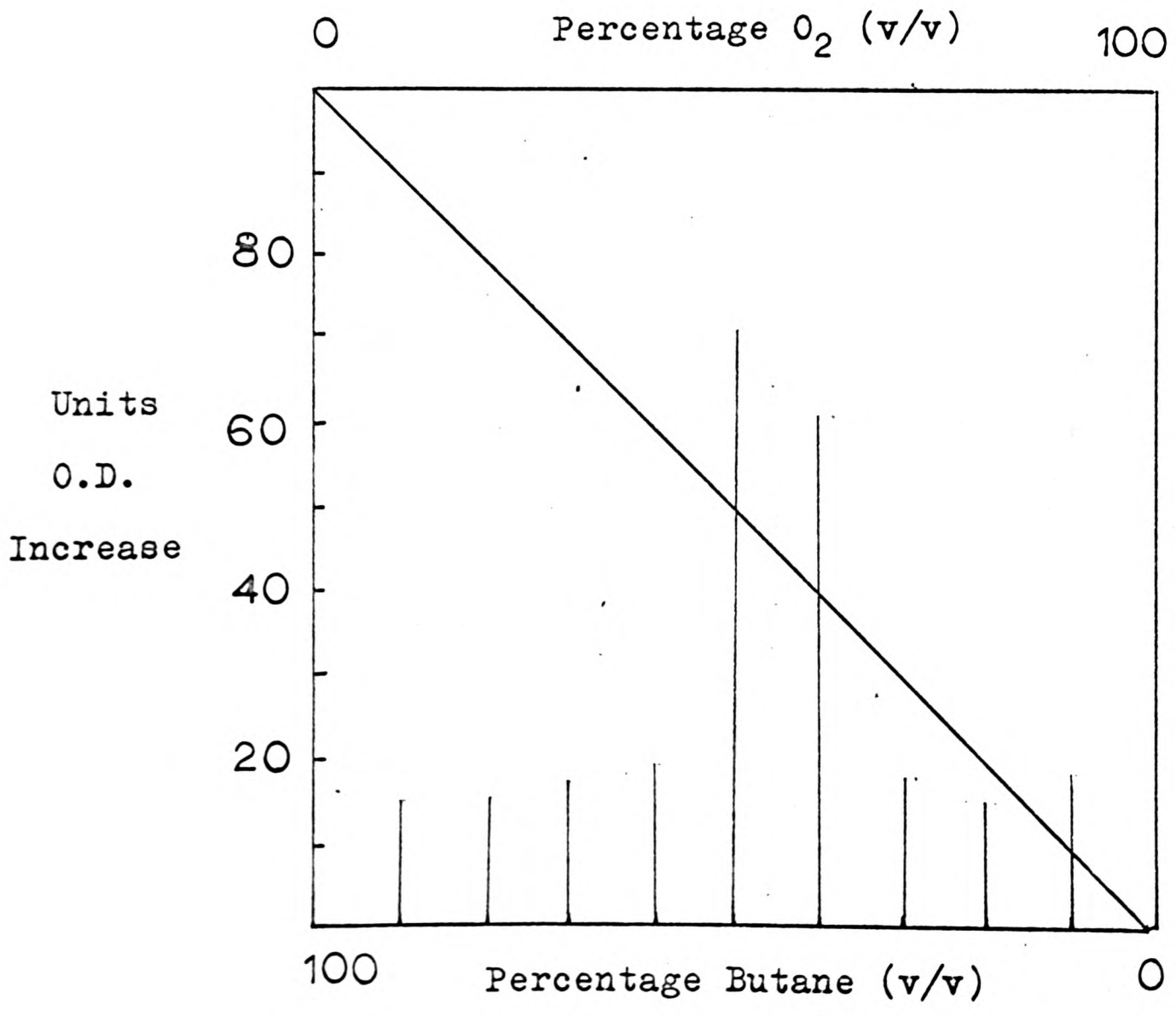


Figure 34

Strain P1

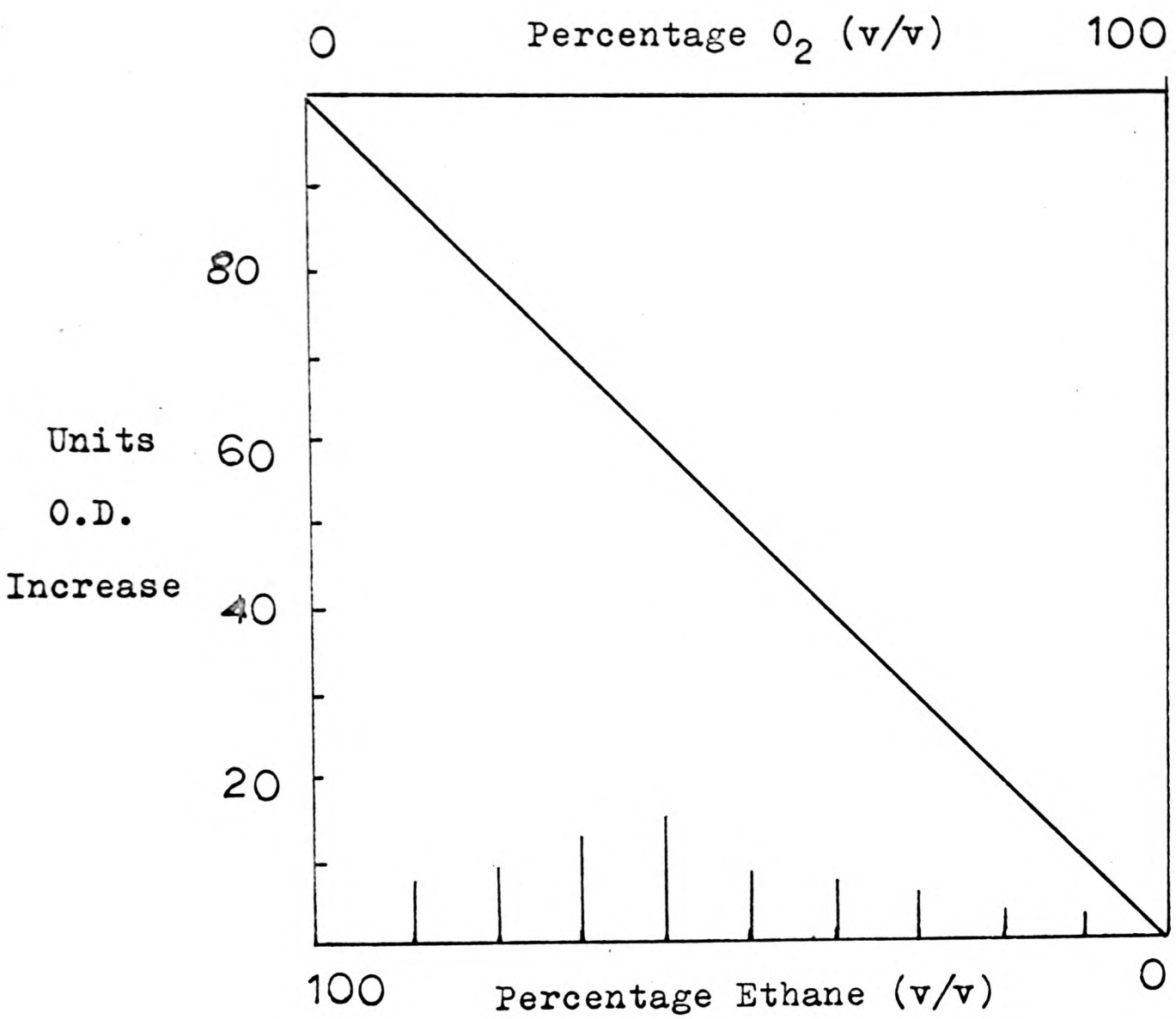


Figure 35

Strain BG28

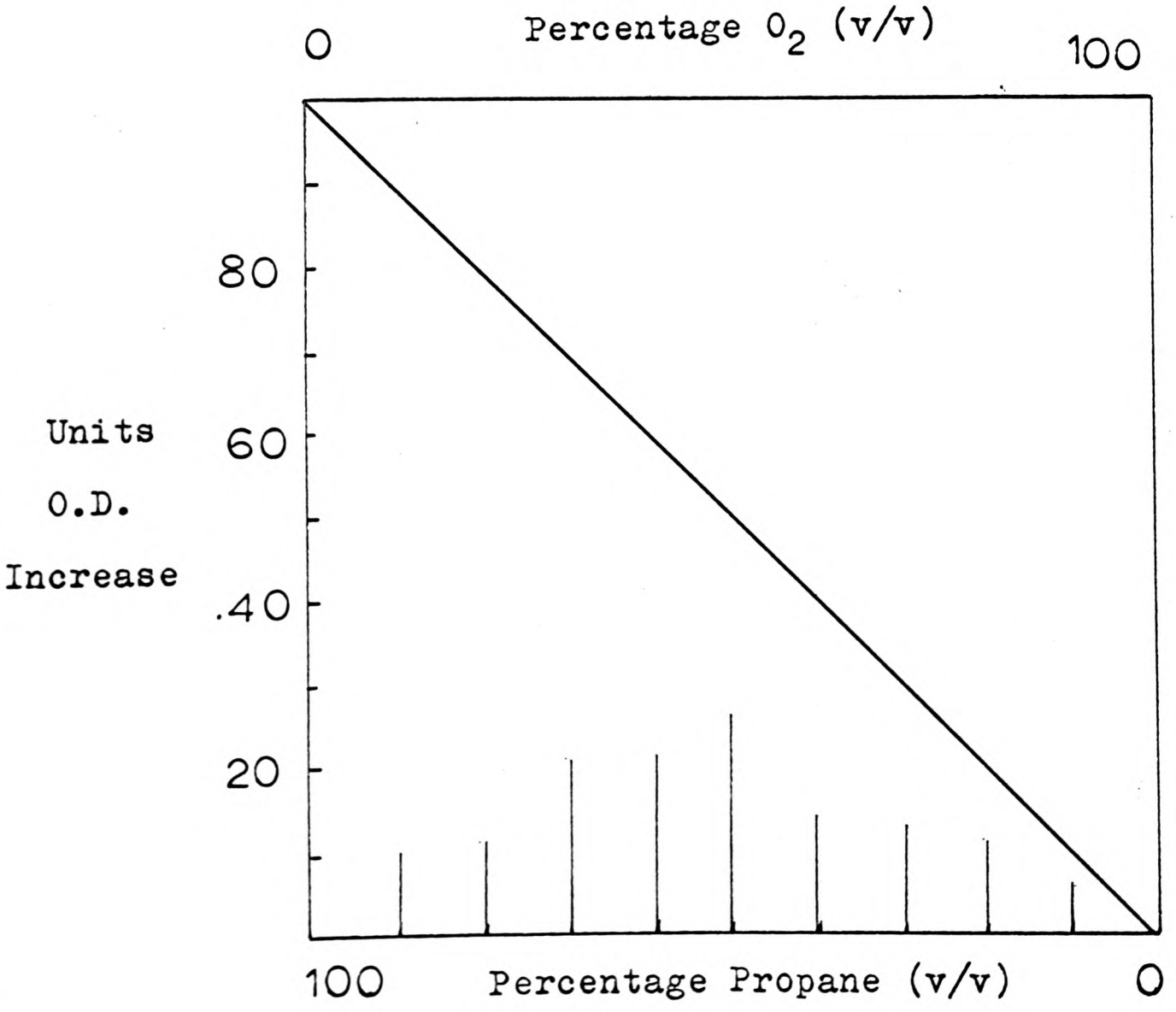


Figure 36

Strain BG28

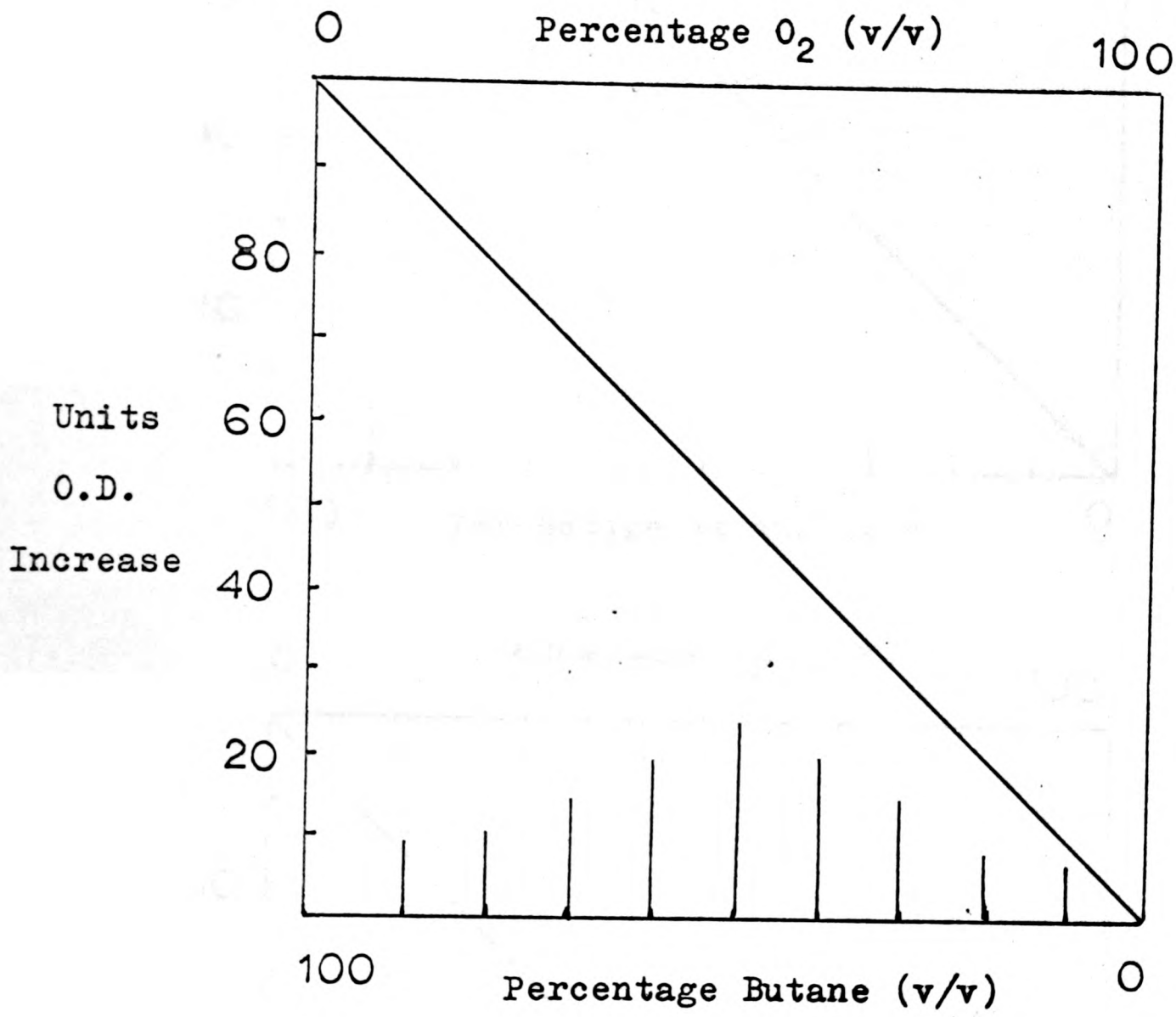


Figure 37

Strain BG28

Growth Rate Studies

A series of experiments was performed to study growth rates of strains P1 and BG28 and to determine optimum growth conditions.

Partial Pressure Optimum

Organisms were grown in static batch culture in sealed test-tubes under different partial pressures of hydrocarbon and oxygen. Results are expressed as units of optical density increase over 5 hours (Figs. 33 to 37).

Maximum growth rate with any hydrocarbon was achieved when the partial pressures of the gas and O_2 were equal. BG28 growing on ethane was the exception having an optimum of 60% (v/v) ethane. Growth rates were observed to increase with hydrocarbon carbon number. This was probably the result of two effects. Firstly, more energy was obtained per molecule of gas metabolised; and secondly, gas solubility increases with carbon number. The latter effect might account for ethane-grown BG28 having an optimum of 60% (v/v), since solubility of ethane at this partial pressure may off-set the growth retarding effect of the lower O_2 partial pressure.

The tendency for growth rate to increase with hydrocarbon partial pressure to a maximum and then to decline indicates an interaction between O_2 and substrate partial pressures. The transfer rate of the hydrocarbon increased with partial pressure, but after 50% (v/v) the decreasing O_2 transfer rate

Table 21

The Effect of Temperature and Nitrogen Source on Growth Rate

Strain BG28

Temperature	Ethane		Propane		Butane	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
22°	11	10	26	26	34	37
25°	17	16	22	29	37	44
30°	19	22	32	29	40	37
34°	30	30	33	31	53	51
37° *						

* Growth too slow to be measured

Table 22

Strain P1

Temperature	Propane		Butane	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-
22°	20	22	22	20
25°	56	56	64	66
30°	51	51	54	55
34°	45	46	47	48
37°	27	27	21	16

Figures represent units of optical density increase over 10 hours

Table 23

Effect of Mixtures of Hydrocarbons on Growth Rate at 30°

Organism	Strain P1	Strain BG28
Hydrocarbons		
Ethane and Propane	37	33
Ethane and Butane	40	26
Propane and Butane	60	42
Ethane, Propane and Butane	47	37

Figures represent units of optical density increase over 10 hours

slowed the growth rate.

Temperature Optimum

The optimum growth temperature for each gas was determined by growing the organisms in their optimum O_2/hydrocarbon atmosphere at the following temperatures: 22, 25, 30, 34 and 37°. Results were expressed as units of optical density increase over 10 hours (Tables 21 and 22). The effect of nitrogen source, ammonium or nitrate ion, was simultaneously measured.

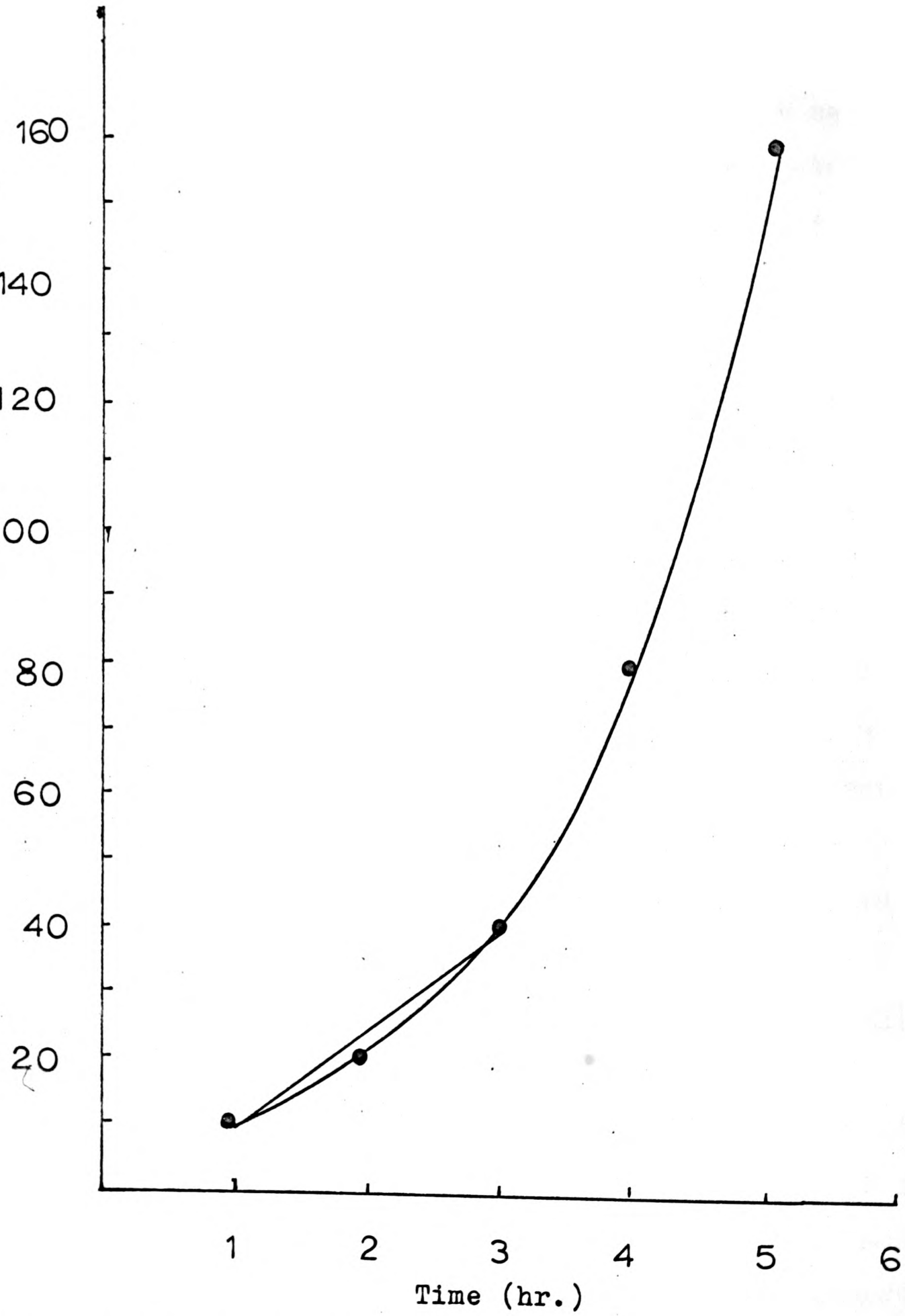
Nitrogen source was shown to have no effect upon growth rates. A difference in optimum temperature between P1 and BG28 was observed, P1 having an optimum of 25° for all gases and BG28 having an optimum of 34°. The fact that the temperature optimum was the same for all gases, together with there being a difference of 9° between the two isolates, would suggest that the influencing effect of temperature was exerted upon the organism itself and not indirectly by varying the solubility of the gases.

Effect on Growth Rate of Mixtures of Hydrocarbons

The growth rates of the isolates were measured in atmospheres of 50% (v/v) O_2 and 50% of two or all three hydrocarbons (hydrocarbons were supplied at equal partial pressures). Rates were measured as increases in optical density over 10 hours (Table 23).

Growth was most rapid when only propane and butane were

Figure 38



Growth Curve of an Organism having a Generation Time of 1 hr., showing Apparent Linearity over Periods of 2 hr.

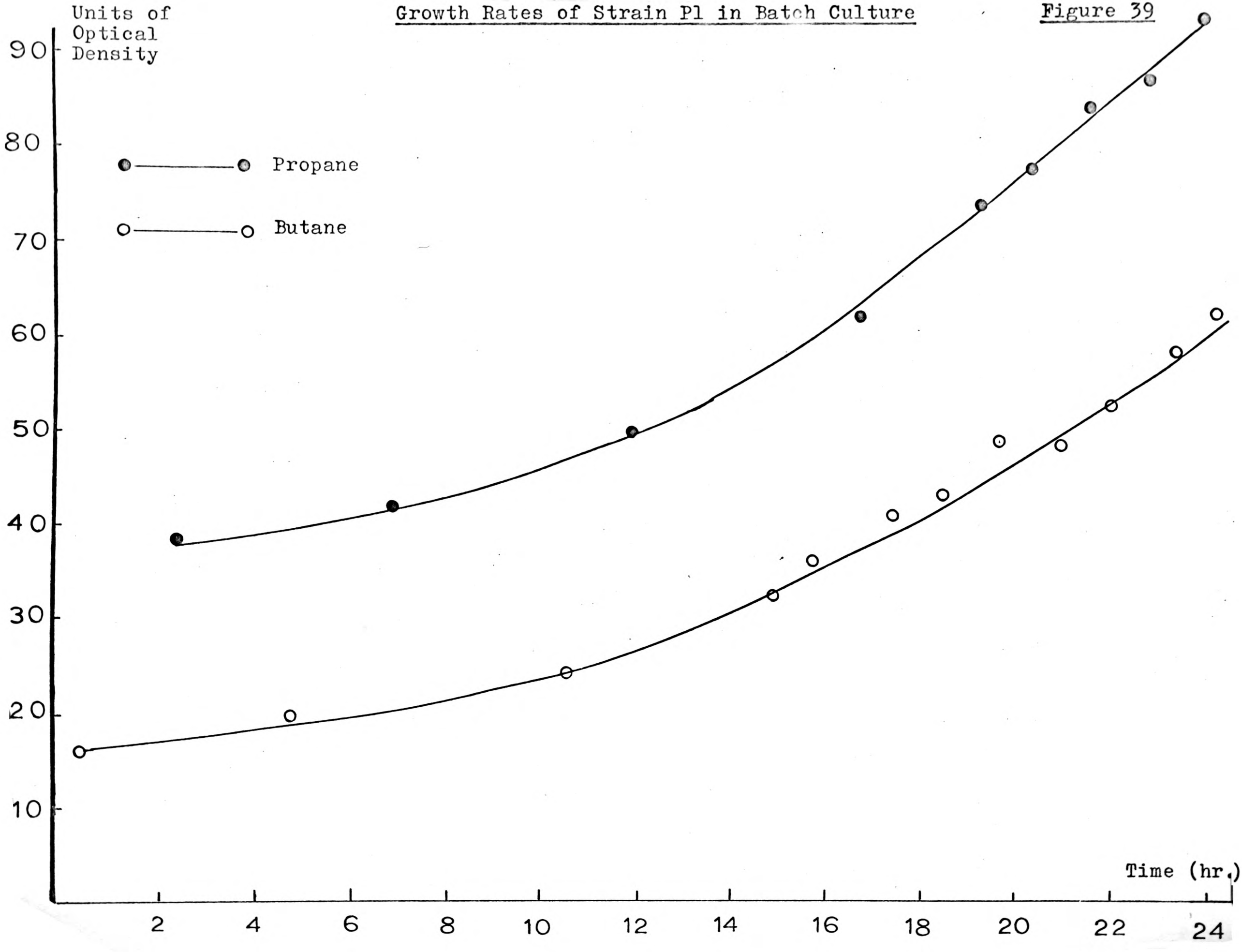
present. If ethane was present with either of these two gases, growth rates were reduced. However, a mixture of all three gases gave better growth than mixtures containing ethane, and only one other gas. This effect may be a result of the production of 2, 3 and 4 carbon fragments, thus relieving the cell of the metabolic burden of synthesising one of these fragments.

Apparent Linear Growth Rates

In all 3 growth rate studies discussed in this section, the growth rates were linear. This might merely have been the result of observing growth for less than two generation times (Fig. 38) or possibly some component of the medium was limiting. The fact that all gradients differed showed that it was the hydrocarbon which was limiting growth. If O_2 had been limiting, all gradients, regardless of the hydrocarbon under investigation, would have been equal. This was verified by growing the isolates in sealed test-tubes in an atmosphere of equal volumes O_2 and N_2 with either 0.1 M acetate, propionate or butyrate as substrate, and observing the growth rates to be different. Thus at 50% (v/v) partial pressure, O_2 was not limiting growth. The linear growth rates observed in static cultures were a result of hydrocarbon limitation due to the variation of the transfer rates at different partial pressures.

Growth Rates of Strain P1 in Batch Culture

Figure 39



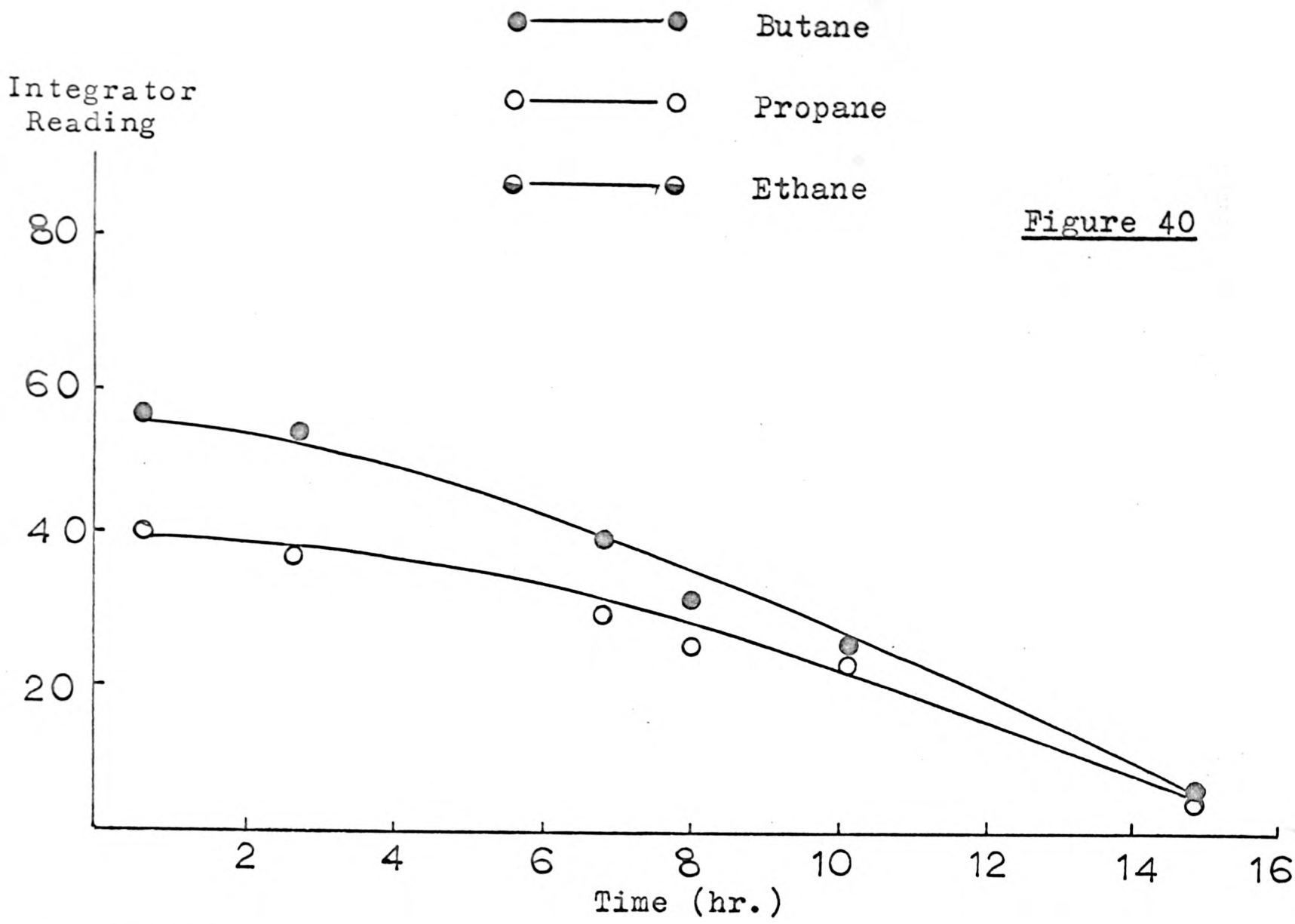


Figure 40

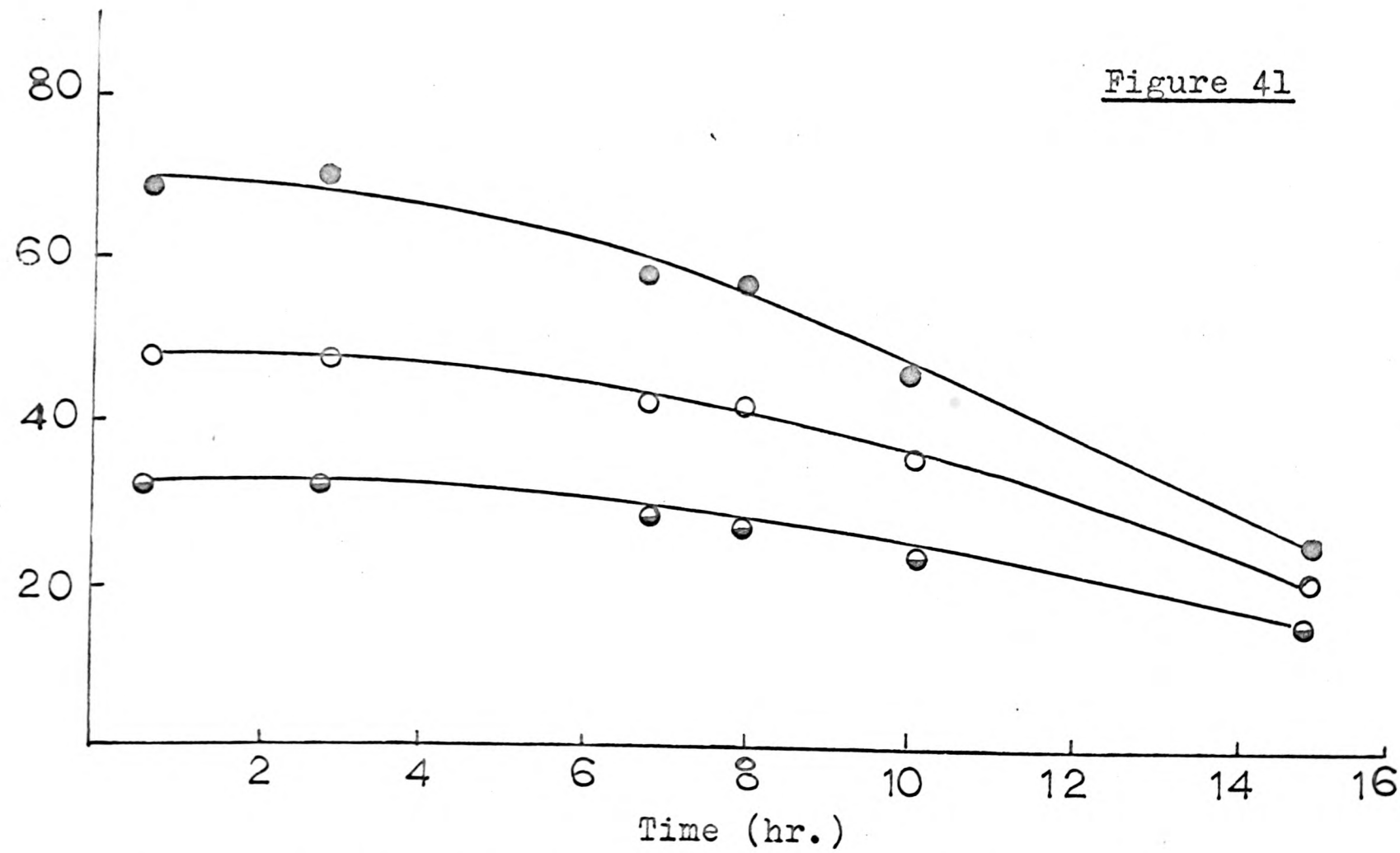


Figure 41

Hydrocarbon Utilization Rates of P1 in Batch Culture

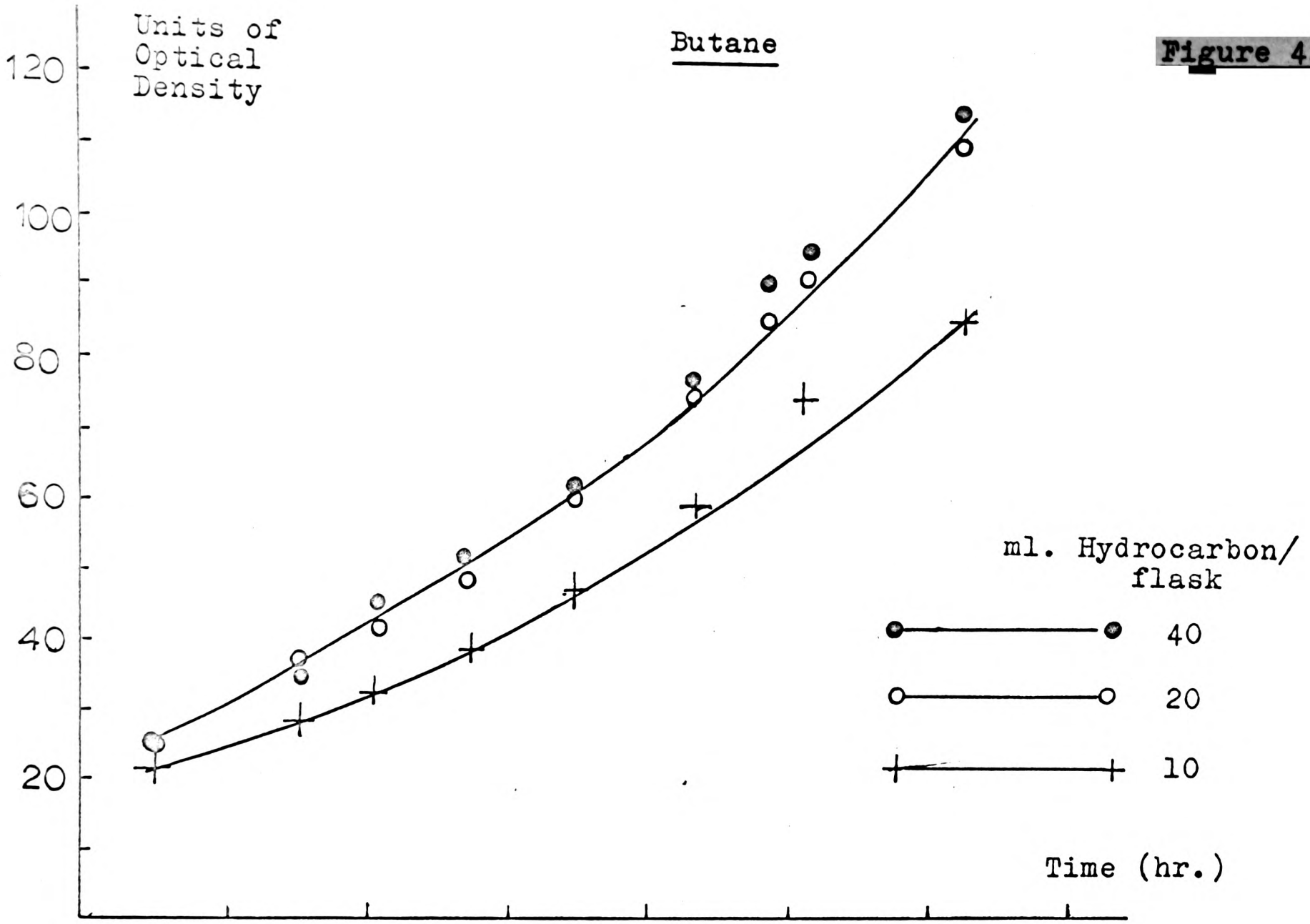
Growth Behaviour in Shake-flasks

As discussed previously, growth could only be measured between 20 and 80 units of optical density, thus it was necessary to grow the organisms in sealed flasks and remove samples periodically, replenishing the gas atmosphere each time. In this way it could be possible to measure optical densities above 80 units. However, the constant opening of the flasks which this procedure involved, so upset growth rates, that this approach was abandoned. Instead, flasks with test-tube side arms were designed which allowed growth to be measured without withdrawing samples, and also the cultures could now be agitated thus removing the effects of gas transfer which may have been limiting growth in the static, sealed test-tubes. Growth rates of P1 growing on propane and butane are shown in Figure 39. Growth over the first 8 hours of measurement appeared to follow the line of an exponential curve, but later readings showed that the growth rate became linear. Similar observations of linear growth rate were made when hydrocarbon uptake rates were measured in batch cultures IN SHAKEN FLASKS (Figs. 40 and 41). During the early stages of growth, the concentration of hydrocarbon in the gaseous phase began to decline exponentially, but later linear uptake was noted. Mueller (1969) also noted a change from exponential to linear growth with methane-utilizing bacteria. This transition occurred as the optical density of the culture increased.

It would seem that growth in batch culture, in a sealed

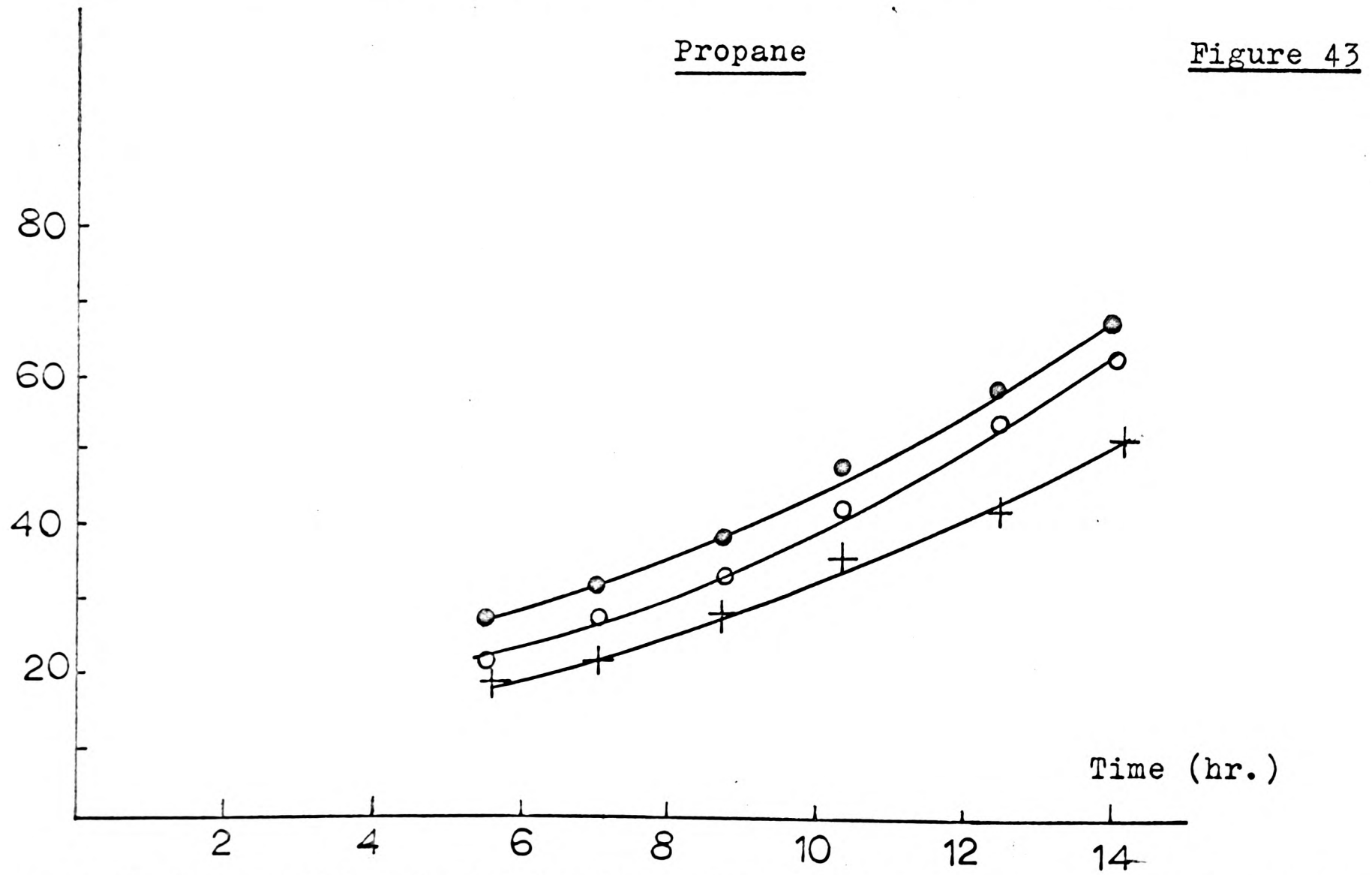
Butane

Figure 42



Propane

Figure 43



Growth Rates of Strain P1 in Differing Partial Pressure of Hydrocarbon

gaseous atmosphere, is exponential in the early stages only, but later becomes linear as the partial pressure of hydrocarbon decreases. As a result, actual generation times were not calculated from the gradients of growth rates, since they are meaningless during linear growth. However, exponential growth in the chemostat with a constant partial pressure of methane and air, occurred with methane-utilizing bacteria (Phillips, private communication). Since the solubilities of methane and the gases used in this report were comparable, there is no reason to suppose that these isolates would behave differently under these same conditions.

The effect of partial pressure upon dissolved gas concentration, and its subsequent effect on growth rate in shake-flasks is shown in Figures 42 and 43. Similar growth rates were obtained for partial pressures above that pressure obtained by adding 20 ml. of hydrocarbon to the flask (about 10% v/v atmospheric pressure). Partial pressures less than this influence growth rate by affecting solubility, i.e. nutrient limitation occurs which slows growth. This implies that growth rate was at a maximum whenever the hydrocarbon partial pressure was greater than 10% of the total. This then suggests that the poor growth rates obtained with gaseous hydrocarbons were not attributable to their low solubilities, but were rather an inherent property of the substrate itself and the metabolism of the organism. Thus growth rates were not increased by increasing the dissolved concentration of the gas

in the medium. (This phenomenon is further discussed in the Industrial Applications Section.)

Effect of Nitrogen Source on Growth Rate

Although experiments involving BG28 and P1 growing in liquid media containing either ammonium or nitrate ions had shown no differences in growth rate (Tables 20 and 21), it was observed that slope cultures of all hydrocarbon-utilizing isolates appeared to grow better on nitrate medium. Slide cultures of P1 and BG28 were made using media containing ammonium or nitrate ions, and growth on butane was observed microscopically.

No difference in the growth rates or the time of mycelial fragmentation was observed. Thus it was concluded that the effect of ammonium ion in reducing growth rate was non-existent during the early stages of growth on solid medium. The slowing of growth rate must then occur when a large mass of cells has been produced on the agar surface. Nitrogen limitation was not operative, since doubling the concentration of the ammonium ion failed to prevent the slower growth rate on agar. It was concluded that the effect was one of a localised rise in pH, which inhibited growth only after several days' production of cells.

Table 24

Preferential Utilization of Gaseous Hydrocarbons Supplied in Equimolar Amounts

Strain P1

Hydrocarbon			Preference Ratio
Ethane	Propane	Butane	
23	52		1/2.25
18		45	1/2.50
	68	77	1/1.14
39	72	81	1/1.85/2.07

Table 25

Strain BG28

Hydrocarbon			Preference Ratio
Ethane	Propane	Butane	
30	43		1/1.44
39		53	1/1.36
	49	56	1/1.14
51	55	63	1/1.08/1.24

Preferential Utilization of a Particular Gas

Pl and BG28 were grown in sealed flasks containing equimolar amounts of 2 or all 3 gases. Utilization of gases was detected by flame ionisation of samples withdrawn periodically. Results are expressed as a percentage of the total gas utilized and also as a preference ratio (Tables 24 and 25).

Both organisms showed a substrate preference ratio which increased with substrate carbon number. With Pl, an organism which grew extremely slowly on ethane, the preference towards propane and butane was more marked when compared with BG28 which does grow on ethane. It would appear that Pl co-oxidises ethane, and presumably assimilates the products, at an appreciable rate only when propane and/or butane are supplied. The extremely slow growth of Pl on ethane alone may be the result of an energy-requiring permease, necessary to obtain this most insoluble hydrocarbon, which only functions when other gases are present to supply the necessary energy. (The mode of entry of gaseous substrates is discussed in the section dealing with lipid content and cellular fine structure.)

With Pl, the substrate preference increasing with carbon number, holds for all series of duplicate or triplicate gas comparisons. However, this relationship between ethane/butane and ethane/propane comparisons, favours propane and not butane with BG28 (Table 24). However, in all other inter-series comparisons, BG28 resembles Pl.

Table 26

Adaption to C_1 - C_4 n-alkanes

Growth Substrate	n-alkanes: Methane	Ethane	Propane	Butane	Growth Substrate
Propane	+	+	+	+	+
Butane	+	+	+	+	+
1-propanol	-	-	+	+	+
1-butanol	-	-	-	-	+
2-propanone	+	+	+	+	+
2-butanone	-	-	-	-	+
2-propanol	+	+	+	+	+
2-butanol	+	+	+	+	+
Propionate	-	-	-	-	+
Butyrate	-	-	-	-	+

Figures show percentage increase of O_2-uptake rate compared to the endogenous rate

Adaption Studies

A series of experiments was performed to study substrate specificity of P1 grown on gaseous hydrocarbons and their possible intermediates. The organism was grown on a particular substrate, resuspended in fresh basal salts medium, and the oxygen uptake rates measured when the other substrates were supplied at 0.01 M concentration. Results were expressed as a percentage increase over the endogenous O_2 uptake rate, and compared to the result obtained when the growth substrate itself was supplied. Although not growth substrates, methane and ethane were also tested.

The results of simultaneous adaption to gaseous hydrocarbons by the organism grown on hydrocarbon and non-hydrocarbon substrates, are shown in Table 26. Bacteria grown on propane or butane were capable of oxidising other gases, even methane which alone did not support growth. This lack of specificity of the oxygenase system was also noted by Perry (1968). Of the non-hydrocarbon substrates tested, bacteria grown on secondary alcohols were also adapted to gaseous hydrocarbons. Bacterial cells adapted to 1-propanol also appeared to oxidise propane and butane. However, the tendency for acetone-grown cells to be adapted to gaseous hydrocarbons was less marked. Results similar to the above have been reported by Perry (1968), who found Brevibacterium cultures grown on 2-propanol or acetone were adapted to gaseous n-alkanes. Also, Lukins (1962), using acetone-adapted

Table 27

Comparative O_2-Uptake Rates of Propane and Butane Oxidation Intermediates

Growth Substrate	Intermediates Tested on Hydrocarbon-grown Cells				
	1-propanol	2-propanone	2-propanol	1-hydroxy-2-propanone	Propionate
Propane 175	305	53	92	130	115
	1-butanol	2-butanone	2-butanol	Butyrate	
Butane 13	283	13	122	136	

Table 28

Acetol as an Intermediate in 2-propanol and 2-propanone Metabolism

Growth Substrate	2-propanol	2-propanone
Growth Substrate Response	17	15
Acetol Response	155	30

Figures represent percentage increase in O_2-uptake rate over the endogenous rate

Mycobacterium strains, noted the presence of an enzyme system for propane oxidation.

There has been much controversy concerning the importance of terminal (C_1) or C_2 as sites of initial oxygen insertion in short chain n-alkanes. Recent findings of Vestal and Perry (1969) have shown that propane was not metabolised by terminal oxidation by their Brevibacterium strain, but rather via acetone and acetol.

From the high rates of O_2 uptake by bacteria supplied with 1-propanol and propionate (Table 27), it is likely that a pathway of propane metabolism proceeding via C_1-oxidation is more operative than one involving C_2-oxidation. Similarly, butane oxidation appears to favour a sequency having 1-butanol and butyrate as intermediates. (The high oxygen uptake rates observed with the secondary alcohols may be the result of non-specific alcohol dehydrogenase activity.) Thus, on the assumption that higher O_2-uptake rates with a particular substrate indicate a predominant metabolic pathway, it would seem that oxygen insertion was at a terminal site in this Nocardia isolate.

Acetol as an Intermediate in 2-propanol and 2-propanone Metabolism

From Table 28, it would appear that acetol was an intermediate in the metabolism of 2-propanol and 2-propanone (acetone). The reason for the poor response of acetone-

adapted bacteria to acetol was obscure. However, it was noted that other substrates also invoked poor responses with these bacteria. Lukins (1962) and Perry (1968) have also shown acetol as an intermediate in acetone metabolism. Vestal and Perry (1969) suggested that acetol was further metabolised to give acetate and CO_2.

Intermediate Accumulation Studies

Strain BG28 was grown on ethane, and strain P1 on propane and butane. Production of metabolic intermediates of these gases was detected by flame-ionisation methods.

Ethanol, acetaldehyde and acetic acid were detected as products of ethane metabolism. Bacteria grown on propane or butane produced 3 detectable peaks after analysis. Two of the 3 were identified as the corresponding primary alcohol and fatty acid. The third could have been either the corresponding methyl ketone or aldehyde, since both had similar retention times on 10% Trimer acid treated Phasepak Q columns. By using Phasepak R, the unknown compound in butane-grown cultures was identified as butyraldehyde. However, acetone and propion-aldehyde remained inseparable on the Phasepak R column. Attempts to demonstrate the presence of acetone by the distillation methods of Lukins (1962) were unsuccessful. It was therefore concluded that the unknown material was propionaldehyde.

The findings of detectable quantities of only the primary

Table 29

Stoicheiometry and Growth Yield Studies with Strain P1

Growth Substrate	Moles O_2/mole Substrate	Moles CO_2/mole Substrate	Percentage (w/w) Yield
Propane	1.60	0.70	37
2-propanone	2.05	1.77	24
1-propanol	2.00	1.17	35
Butane	1.98	0.87	47
2-butanone	2.58	1.88	22
1-butanol	1.73	1.42	20

alcohol, aldehyde and fatty acid in these cultures further supports the suggestion that the primary site of oxygen insertion by Nocardia is the C_1-position.

Stoicheiometry and Growth Yield Studies

Strain P1 was grown on propane, n-propanol and 2-propanone; and butane, n-butanol and 2-butanone. Oxygen uptake, carbon dioxide production and dry weight yields were determined (Table 29).

Conversion of substrate into cellular material was more efficient with the gases than with their corresponding intermediates. This is reflected in the proportionally smaller amount of CO_2 produced with gaseous substrates. Blevins and Perry (private communication) reported 40-50% (w/w) cell yields on gaseous hydrocarbons. However, they found that yields on corresponding alcohols and fatty acids were 60-75% (w/w) higher.

The poorer yields on alcohols and methyl ketones, together with the high molar ratios of O_2 used and CO_2 produced, obtained here, suggested that some non-assimilative oxidation of substrate or storage compounds might have occurred. This is unlikely with the methyl ketones, since 30-40% of the substrate was unused at the time of harvesting.

The higher yield of organisms obtained with butane compared with propane agrees with the findings of others: that yield improves with chainlength. This could be accounted for if a NADH-dependant, mixed function oxidase system was involved

as the initial oxygen insertion mechanism. The compensation (in terms of available carbon atoms) for the loss of one molecule of reduced NAD would be much greater (33%) if butane were the substrate and not propane. This effect would become less noticeable as carbon number increased.

Because of the reduced nature of hydrocarbons, the insertion of oxygen has the effect of raising the yield factor above that obtained for the corresponding alcohol.

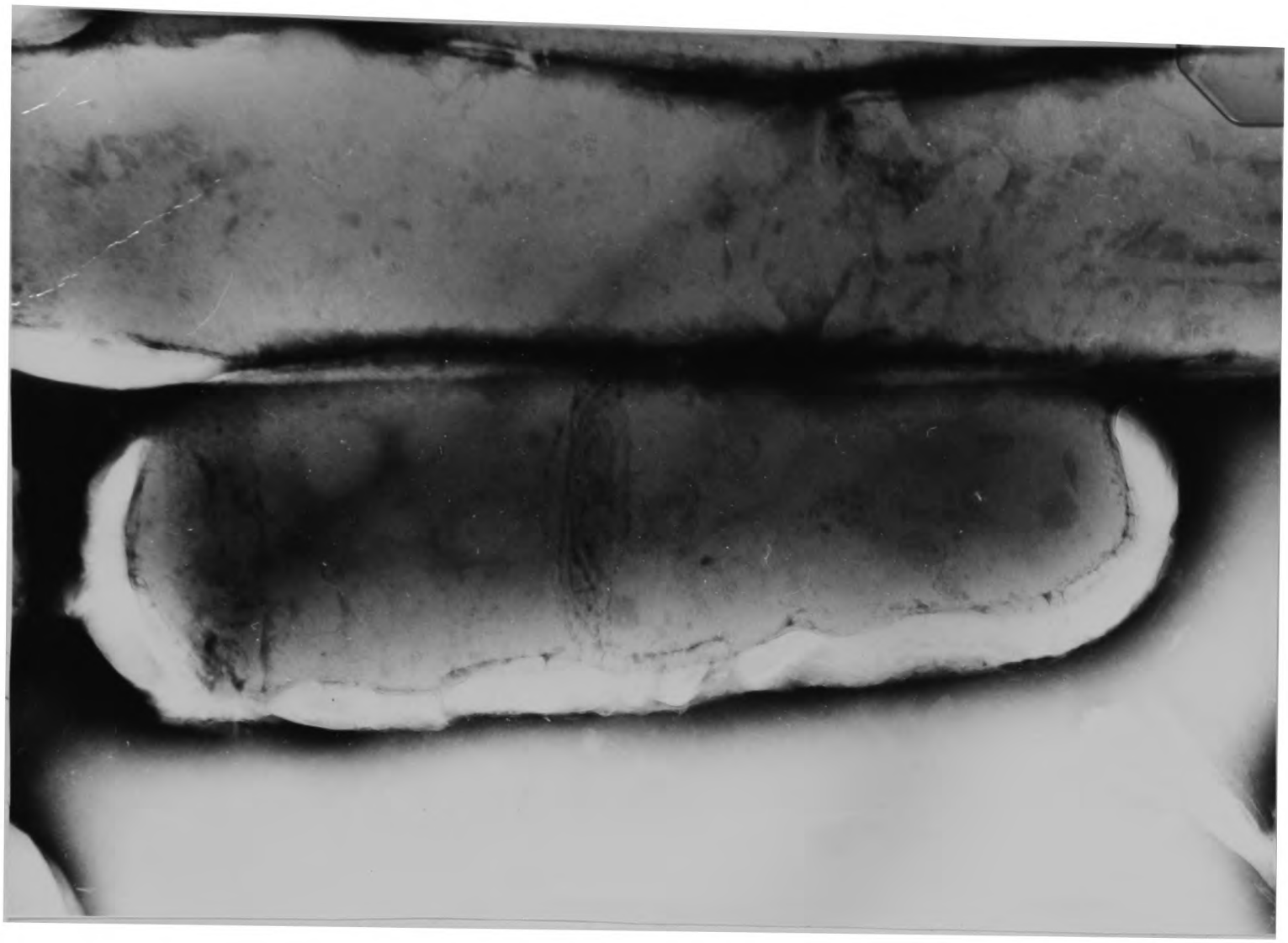


Figure 44

Glucose-grown

Strain P1

Figure 45

Propane-grown

Strain P1

Magnification x 60,000

Figure 46

Propane-grown

Strain P1

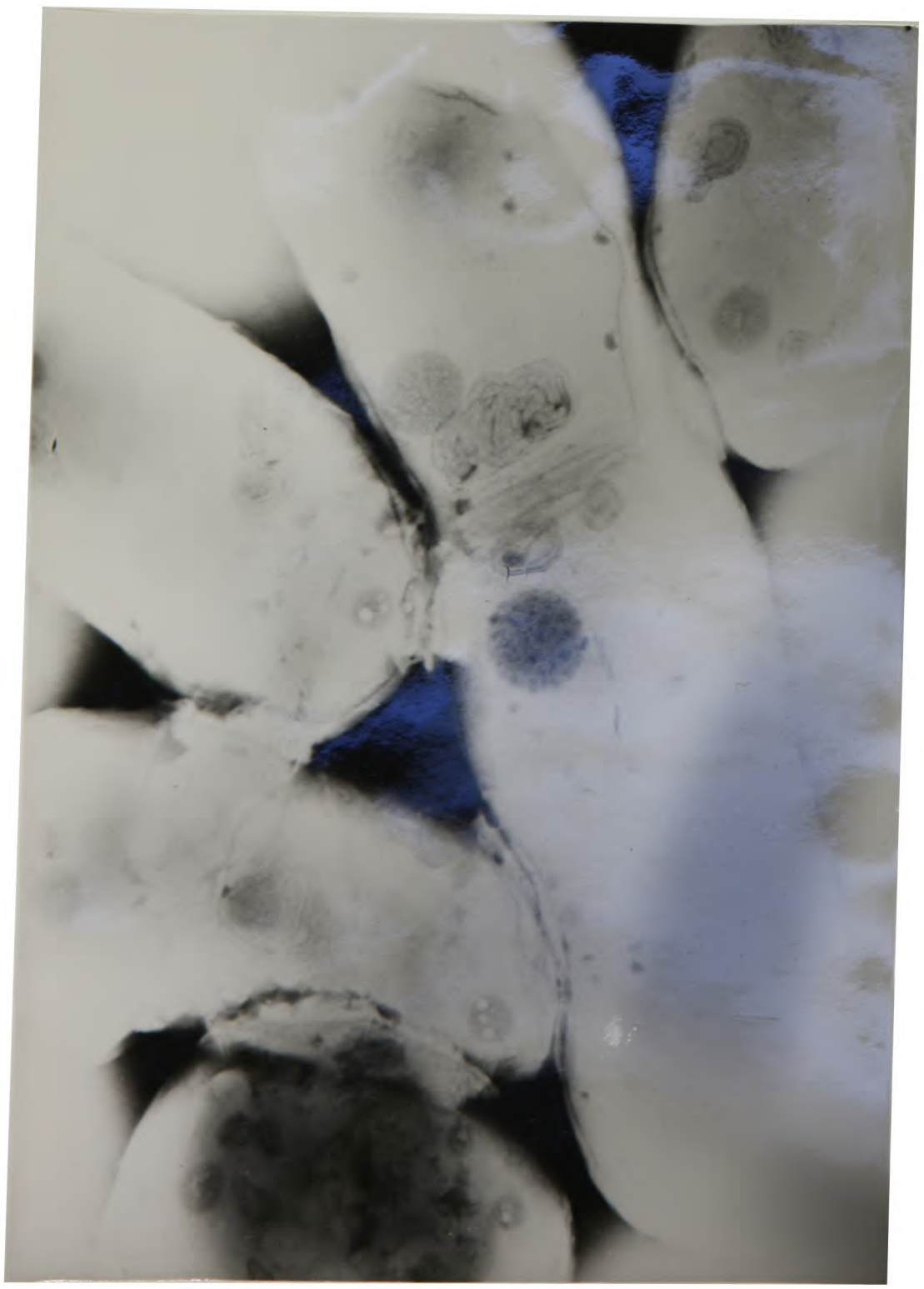


Figure 47

Propane-grown

Strain P1

Magnification x 60,000

Table 30

Percentage Dry Weight of Lipid Material of Strain P1

Growth Substrate	Ether-Soluble Fraction	Ether-Insoluble Fraction	Total Lipid
1-propanol	11.5	17.0	28.5
Propane	12.5	10.5	23.0
1-butanol	6.6	8.8	15.4
n-butane	7.6	17.0	24.6

Results are expressed as a percentage (w/w)
of total cell dry weight

Mode of Assimilation of Gaseous Hydrocarbons

It has been suggested that the high lipid content of hydrocarbon-utilizing micro-organisms played a role in the assimilation of hydrocarbon from the aqueous environment. Davies and Whittenbury (1969) observed complex membranous systems in methane-utilizing bacteria, and have suggested that they probably act as sites for respiratory enzymes, and that the lipid content may serve as an intracellular "pool" in which the methane was preferentially soluble.

Electron Microscopy

Sections of strain P1 were examined on the electron microscope for internal membranes, the organism having been grown on propane and glucose (Figs. 44 to 47). Bacteria grown on both substrates had small membranous regions at the cross-walls, but only hydrocarbon-grown cells had small mesosome-like structures along the longitudinal walls. These structures were very primitive in comparison with the structures of methane-utilizing bacteria.

Lipid Contents

Analyses of the lipid content of strain P1 cells grown on propane, butane and the corresponding primary alcohols are shown in Table 30. There was little difference between the amount of total lipid in 1-propanol and propane-grown cells, but the ether-insoluble fraction of butane-grown cells was

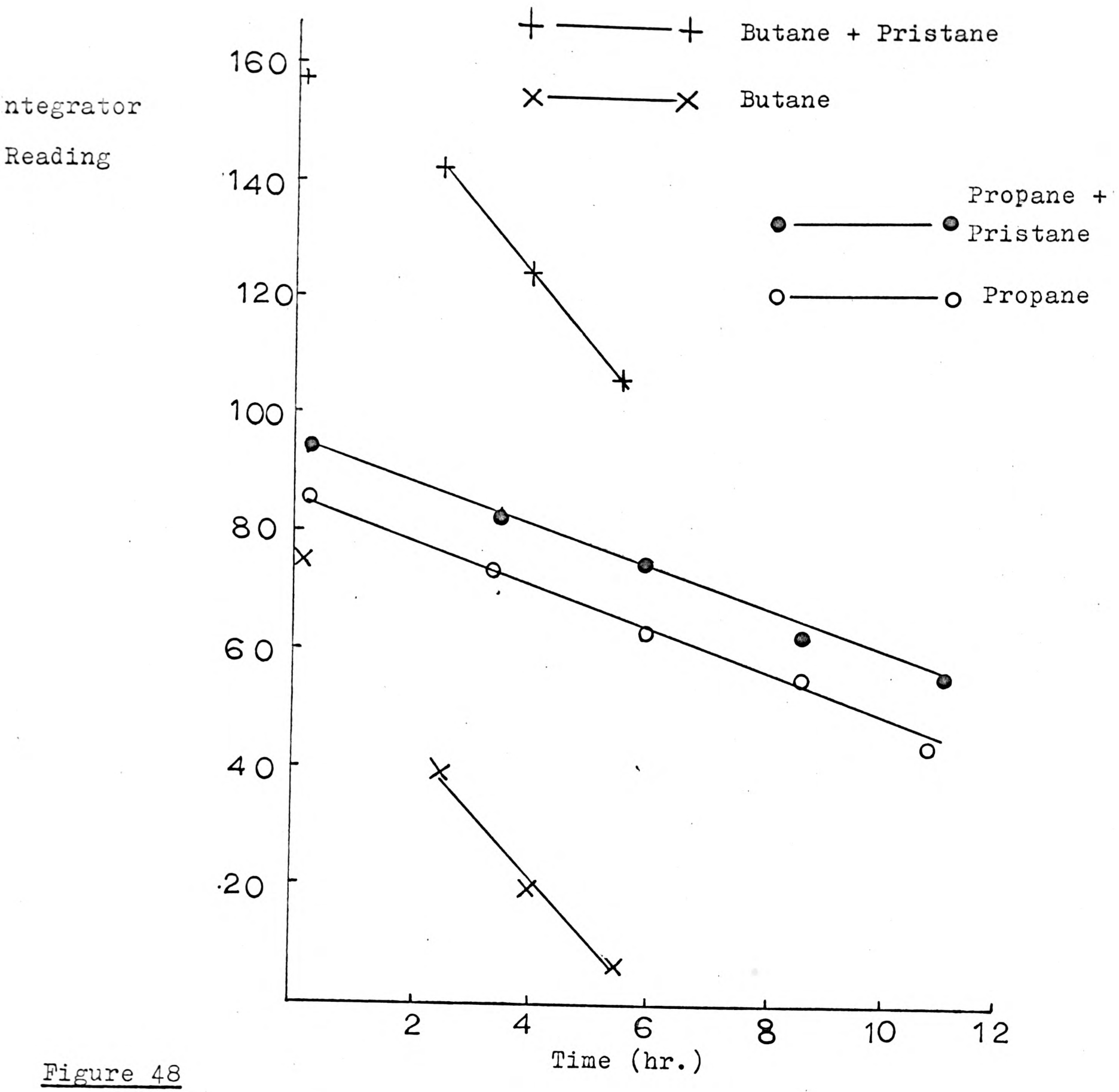


Figure 48

Rates of Hydrocarbon Uptake by Strain P1 in the Presence or Absence of Pristane

nearly twice that of 1-butanol-grown cells. This is reflected when the total lipid contents were compared.

Hot acetone extraction of all ether-insoluble fractions showed traces of waxes when cooling. (This was particularly so with butane-grown cells.) Similarly, chloroform treatment of all ether-insoluble fractions showed these waxes as insoluble residues. It was assumed that the chloroform-soluble material was poly-β-hydroxybutyrate. (The high lipid content of these hydrocarbon-grown cells is further discussed in the section on Industrial Applications.)

In the following experiments the importance of lipid in dissolving hydrocarbons and assisting in growth, is examined.

Pristane Studies

An experiment was performed to determine if cells growing in lipid droplets (pristane) utilized propane and butane more rapidly than cells growing in the aqueous phase. A more rapid gas uptake in the presence of pristane would suggest that the dissolved gas in the pristane was more available to the cell.

P1 was grown in sealed flasks in the presence or absence of pristane, and the uptake rates of the hydrocarbons were measured using flame-ionisation detection methods. Bacteria growing with pristane became attached to the droplets, leaving the aqueous phase clear. Comparative gas uptake rates are shown in Figure 48. Rates were unaffected by pristane.

Gas Uptake by Non-proliferating Cells

The uptake of gases by wet pellets of heat- or formalin-killed P1 cells, was examined. Bacteria were placed in a test-tube, which was sealed, and the uptake of the gases was measured by flame-ionisation method. As a control, the gas uptake of a similar volume of water was also measured.

No gas was taken up by any of the bacterial samples.

Gas Uptake by Freeze-dried Cells

Samples (0.1 g.) of freeze-dried strain P1 cells which had been grown on 1-butanol, 1-propanol, propane and butane were placed in sealed test-tubes and the rates of hydrocarbon uptake measured.

No gas uptake was observed in any of the samples.

As a result of the above three experiments, it was concluded that lipids played no significatn part in the cellular assimilation of dissolved hydrocarbons. It is likely that the membranous systems in these hydrocarbon-grown cells act as sites for respiratory enzymes and permeases necessary to obtain sufficient quantities of substrate from the dilute aqueous solution.

Industrial Implications

The prime prerequisites of the commercial production of unicellular biomass are that the organism grows quickly, and gives high yields of protein-rich cells. Other factors are

important, e.g. digestibility, ease of harvesting, culture stability, etc., but these are only of secondary significance to be screened after an organism has been found to satisfy the primary criteria.

In this report a variety of organisms have been examined for their ability to grow rapidly on gaseous hydrocarbons, but only two isolates have been found suitable. Growth was most rapid on butane, with the rate on other gases decreasing with carbon number. Although it was impossible to study exponential growth to obtain actual generation times in closed batch culture, it was observed that an increase in hydrocarbon partial pressure over a certain pressure failed to hasten growth. This strongly suggests that the growth rate observed on any particular gas is uninfluenced by dissolved hydrocarbon concentration, but is merely the optimum performance of which the organism is capable. At high population densities in an industrial fermentor, the transfer of hydrocarbon to the aqueous phase may not occur rapidly enough to maintain this fixed growth rate, and as a result substrate limitation may occur. Due to their more rapid transfer rates the hydrocarbons used in this report appear more suitable substrates than methane. Mass transfer could be improved by designing fermentors in which the bubbles of gas were submerged for as long a period as possible. Also, separate addition of the air and hydrocarbon, the concentration gradient between gas bubble and liquid would be greater, thus allowing a more rapid

transfer of substrate. This would be accentuated at high cell densities when the dissolved gas concentration was low.

The observation that growth was rapid on a mixture of gases is encouraging, since it would be as a gaseous hydrocarbon fraction that the substrate would be supplied industrially. However, the problem remains one of supplying a gaseous substrate. Even if gases were recycled through a single fermentor, or if a series of such fermentors were used, more capital equipment would be involved than if liquid n-alkanes were used.

Yields on gaseous hydrocarbons were found to be low when compared with yeasts growing on liquid paraffin. An extensive isolation survey may supply a bacterium which is more efficient at converting substrate into cell material. However, such a study might be better directed towards isolating a yeast, since these organisms are likely to contain less lipid than those bacteria normally regarded as being the most suitable for biomass production. Other reasons for preferring yeasts would be reduced harvesting costs; a low pH optimum, avoiding contamination problems and the likelihood of a more digestible, less toxic, nutritional source. However, it may be that no yeasts will grow on gaseous hydrocarbons.

In conclusion, it would seem that the biological, physiochemical and economic problems besetting biomass production from gaseous hydrocarbons do not warrant the present enthusiasm for these substrates.

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